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A novel locust (*Schistocerca gregaria)* serine protease inhibitor with a high affinity for neutrophil elastase

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Abbreviations

PPE, porcine pancreatic elastase; HNE, human neutrophil elastase; ; Abz, *O*-aminobenzoyl; EDDnp, *N*-(2,4-dinitrophenyl) ethylenediamine; Igepal CA-630, (octylphenoxy)polyethoxyethanol;; OMTKY-III, turkey ovomucoid trypsin inhibitor third domain; α_1-Pi, human α_1- protease inhibitor; SLPI, human secretory leucocyte protease inhibitor; DEAE, diethylaminoethyl; *p*-NA, *p*-nitroanilide ; rp-h.p.l.c, reverse phase high pressure liquid chromatography ; TBE, thiobenzyl ester

Running Title

A potent locust neutrophil elastase inhibitor

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Synopsis (230 words) : We have purified to homogeneity two forms of a new serine protease inhibitor specific for elastase/chymotrypsin from the ovary gland of the desert locust *Schistocerca gregaria*. This protein, greglin, has 83 amino acid residues and bears putative phosphorylation sites. Amino acid sequence alignments revealed no homology with pacifastin insect inhibitors and only a distant relationship with Kazal-type inhibitors. This was confirmed by computer-based structural studies. The most closely related homologue is a putative gene product from *Ciona intestinalis* with which it shares 38% sequence homology. Greglin is a fast-acting and tight binding inhibitor of human neutrophil elastase (k_{ass} = 1.2 x 10^7 $M^{-1}s^{-1}$; K_i = 3.6 nM) and subtilisin. It also bind neutrophil cathepsin G, pancreatic elastase and chymotrypsin with a lower affinity (26 nM ≤ K_i ≤ 153 nM), but do not inhibit neutrophil protease 3 or pancreatic trypsin. The capacity of greglin to inhibit neutrophil elastase was not significantly affected by exposure to acetonitrile, high temperature (90°C), low or high pH (2.5-11.0), N-chlorosuccinimide-mediated oxidation or the proteolytic enzymes trypsin, papain and pseudolysin from *Pseudomonas aeruginosa.*

Greglin efficiently inhibits the neutrophil elastase activity of sputum supernatants from cystic fibrosis patients. Its biological function in the locust ovary gland is presently unknown, but its physicochemical properties suggest that it can be used as a template to design a new generation of highly resistant elastase inhibitors for treating inflammatory diseases.

Key words: serine protease; kinetics; kazal inhibitor; neutrophil elastase; insect.

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INTRODUCTION

Neutrophil elastase is thought to be the major protease involved in tissue destruction during inflammation. This is especially true for lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD), and acute respiratory distress syndrome (ARDS), which are accompanied by considerable recruitment of neutrophils. Anti-inflammatory therapy using protease inhibitors may be an efficient means of reducing the unopposed proteolytic activity (e.g HNE activity) that occurs when neutrophils invade inflammatory sites (reviewed in [1]). A number of studies using natural and recombinant inhibitors have had mixed results due to ineffective administration, or the inactivation of the inhibitor by mechanical or physicochemical constraints in the local environment [1]. There is thus a need for molecular structures that possess the physicochemical and mechanical properties required for use *in vivo*.

At least 2500 sequences homologous to those of known protease inhibitors can be retrieved from data banks, and assigned to one of 48 families [2]. The classical inhibitors of HNE and other neutrophil proteases are members of families I1 (Kazal), I2, I3, (Kunitz), I4 (serpin), I17 (elafin). The most prominent physiological inhibitor of HNE is α_1-Pi (I4 family), the recombinant form of which has been given by inhalation or intravenously to treat cystic fibrosis and α1-Pi deficiency. Several other inhibitors, including MNEI, SLPI and elafin/pre-elafin, have also been given as aerosols or systemically to inhibit neutrophil elastase. Most of these natural inhibitors are sensitive to oxidation and/or proteolytic degradation by other proteases that are present at inflammatory sites [1]. Recombinant protease inhibitors based on the sequences of natural endogenous inhibitors have been developed to overcome some of these drawbacks and a few have proved to have beneficial anti-inflammatory properties [3].

Synthetic low Mr elastase inhibitors have also been developed, but their side effects and limited efficacy are barriers to further work on them [1].

Insects have many more serine proteases than do humans, but they have relatively few serine protease inhibitors [4] [5]. A new family of low Mr protease inhibitors, the pacifastins, was found recently in arthropods [6].They all have 6 cysteine residues arranged in a conserved disulfide pattern [6]. Most of them preferentially inhibit chymotrypsin and trypsin. Five pacifastin inhibitors have been purified from the ovary of the desert locust *Schistocerca gregaria*

[7]. We have now isolated and purified to homogeneity two forms of a novel inhibitor of higher Mr, greglin, from the same material. The protein is a powerful neutrophil elastase inhibitor that also inhibits pancreatic elastase and chymotrypsin-like proteases.

We used computational tools to look for sequence homologies with other proteinaceous inhibitors and to predict the secondary and tertiary structures of greglin.

EXPERIMENTAL

Materials

Schistocerca gregaria ovaries were obtained as previously described [7, 8]. Porcine pancreatic elastase (PPE; EC 3.4.21.36), human neutrophil elastase (HNE; EC 3.4.21.31), protease 3 (EC 3.4.21.76), cathepsin G (EC 3.4.21.20) and human α_1-Pi were purified [9, 10] or obtained from Athens Research and Technology (Athens, GA, USA). Porcine pancreatic trypsin (EC 3.4.21.4) and subtilisin Carlsberg (EC 3.4.21.62) were obtained from Sigma (St Quentin Fallavier, France). Bovine pancreatic chymotrypsin (EC 3.4.21.1) was purchased from Worthington (Lakewood, NJ, USA) and papain (EC 3.4.22.2) from Roche Diagnostics (Meylan, France). The active sites of all the above proteases were titrated using published methods [11 , 12-14]. The concentrations given here refer to active protein concentrations. Pseudolysin (E.C 3.4.24.26), endoprotease Glu-C (EC 3.4.21.19) were from Calbiochem (VWR, Strasbourg, France) and Sigma respectively. Succinyl-Ala-Ala-Ala-*p*-nitroanilide [Suc-$(Ala)_3$-*p*-NA], methoxysuccinyl-Ala-Ala-Pro-Ala-thiobenzyl ester [MeOsuc-$(Ala)_2$-Pro-Ala-TBE], benzoyl-Arg-*p*-nitroanilide (BAPNA), succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide [Suc-$(Ala)_2$-Pro-Phe-*p*-NA] and methoxysuccinyl-Lys(2-picolinoyl)-Ala-Pro-Val-*p*-nitroanilide [MeOsuc-Lys-(pico)-Ala-Pro-Val-*p*-NA, the chromogenic substrates of the various proteases were purchased from Bachem (Bubendorf, Switzerland). The HNE fluorogenic substrate Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp (Abz-APEEIMRRQ-EDDnp) was used as described [15].

Sputum samples were collected from adult cystic fibrosis patients after informed consent. Approval was obtained from the Ethics Committee of our Institution.

Purification of greglin

The detailed purification procedure is reported in the “Supplementary material section”. Briefly, an homogenate of 150 *S gregaria* ovaries was precipitated between 50-80% solid ammonium

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sulphate and the resulting pellet suspended and fractionated by gel filtration. The collected fractions were checked for their anti-elastase activity using Suc-$(Ala)_3$-*p*-NA as a substrate. The elastase-inhibiting fractions were pooled and chromatographed on an anion exchange column. The bound material was eluted with 0 to 300 mM gradient of NaCl and the fractions containing inhibitor detected as above and further chromatographed on a Mono Q HR 5/5 column. The two major peaks were further purified by chromatography on the same column and their purity checked by SDS gel (12%) electrophoresis and rp-h.p.l.c. using a C4 Brownlee cartridge.

Kinetic measurements

Experimental details related to the measurements of enzymes activities on chromogenic and fluorogenic substrates are given in the "Supplementary material" section.

Determination of active inhibitor concentrations

The active concentration of each inhibitor was determined with active site-titrated HNE. Increasing quantities of inhibitor were incubated with 0.38 μM enzyme in 990 μl reaction mixtures for 10 min. The remaining enzyme activities were measured by adding 10 μl 50 mM Suc-$(Ala)_3$-*p*-NA. Under these conditions the decrease in HNE activity as a function of both inhibitor concentrations was linear up to about 90 % inhibition. We used the linear portion of the inhibition curve to deduce the active concentration of both inhibitory species assuming a 1 : 1 binding stoichiometry.

Determination of $K_{i,}$ the equilibrium dissociation constant for the complexes formed between greglin and various proteases

*C*onstant amounts of a given protease were reacted with increasing amounts of inhibitor in 990 μL buffered reaction mixtures for 20 min. The remaining enzymic activities were measured by adding 10 μL of appropriate substrate solution [16]. Further details are given in the "Supplementary material" section.

Determination of k_{ass} and k_{diss}, the association and dissociation rate constants for the interaction of HNE with greglin

The association and dissociation kinetics for the reaction of HNE with greglin 1 and 2 were investigated by the progress curve method [16] using a stopped-flow apparatus (SFM3, Bio-

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Logic, Claix, France) [16, 17]. Data acquisition and processing were done with the manufacturer's BioKine software.

Assessment of the residual greglin inhibitory activity after incubation with non-target proteolytic enzymes

2.8 nM HNE was incubated with 14 nM native or protease-treated inhibitor for 10 min at 37 ° C in 212 µL buffer. The residual enzyme activity was measured by adding 2 µl 0.2 mM Abz-APEEIMRRQ-EDDnp.

Inhibition by greglin of the HNE activity in cystic fibrosis sputum supernatant

The sputum samples collected from adult cystic fibrosis patients were homogenized in 10 mM phosphate-buffered saline (PBS), pH 7.4 (3 mL/g sputum) and centrifuged at 10,000 x g for 10 min at 4°C. The concentrations of active HNE in these samples were deduced by comparing their enzyme activities with that of pure, active-site-titrated HNE using Abz-APEEIMRRQ-EDDnp (1.33 µM final). Buffered reaction mixtures containing greglin (3 to 36 nM final) were incubated with samples of sputum supernatant containing HNE (30 nM final) for 10 min. at 37°C. Residual HNE activity was measured spectrofluorometrically using Abz-APEEIMRRQ-EDDnp as substrate.

Determination of the primary structure of greglin

The complete amino acid sequences of the two reduced and alkylated greglin isoforms were determined by automated N-terminal sequencing of the purified inhibitors and the enzymatically and chemically cleaved forms using an Applied Biosystems Procise pulsed liquid sequencer with the chemicals and program recommended by the manufacturer. Inhibitor (1 nanomol) was reduced with dithiothreitol and alkylated with 2 µL 4-vinyl pyridine (see Supplementary Materials). Samples were desalted by rp-h.p.l.c. and incubated (final concentration: 4.6 nM) with trypsin (2.0 nM final) or chymotrypsin (1.5 nM final) in appropriate buffers. The products were separated by rp-h.p.l.c, freeze-dried and sequenced. .

The reduced, alkylated inhibitors (1.5 nM final) were also incubated in the dark at 20°C for 18 hours with 75 mg/mL cyanogen bromide in 70 % formic acid. The products were separated by rp-h.p.l.c, freeze-dried and sequenced.

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C-terminal sequences were determined by mass spectrometry using a Bruker BIFLEX III ™ mass spectrometer (Bremen, Germany) in linear positive ion mode. Samples were prepared by the sandwich method [18]. Purified peptides were incubated at 25 °C for 3 hours with 10 ng/µL carboxypeptidase P (Sigma) in 10 µL 50 mM sodium citrate buffer , pH 4.0.

Prediction of the secondary and tertiary structures of greglin

Sequence analysis tools

The BLAST (*http://www.ebi.ac.uk/blastall/*) and MEROPS (*http://merops.sanger.ac.uk/*) suites of programs were used to look for homologies in the sequence databases. We used T-coffee (*http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi*) to generate multiple sequence alignments, and NetPhos (*http://www.cbs.dtu.dk/services/NetPhos/*) to predict phosphorylation sites.

Secondary structure prediction

Seconday structural elements were predicted using sspro[19], nnpredict [20], psipred, sam, jufo through the Robetta server [21], sable and profsec through the GeneSilico metaserver (*http://genesilico.pl/meta*).

These programs were selected to cover the whole range of methods (neural network, hidden Markov chain, position specific and profile matrices, etc.) and parameters (solvent representation, amino acid properties, etc.) that are currently available.

Tertiary structure prediction

To predict the tertiary structure of greglin, we used the automated GeneSilico metaserver (*http://genesilico.pl/meta*) that uses results from the inbgu, 3dpssm, ffas, mgenthreader, sam, sparks, fugues, and 3dpssm servers. The Robetta server was used to build the structure of greglin starting from its primary sequence alone [21].

SwissPdbViewer (*http://au.expasy.org/spdbv/*) was used to generate multiple structural alignments and to superimpose themodels obtained.

Greglin physicochemical properties

Oxidation by N-chlorosuccinimide

Greglin (13 µM final) was incubated at room temperature with N-chlorosuccinimide (125 µM final) in 50mM Hepes buffer pH 7.4, 150 mM NaCl, 0.05% Igepal CA-630 for 20 min. Excess

oxidant was removed by rp-h.p.l.c. on a C4 column and oxidation was checked by mass spectrometry using a M@LDI L/R Waters micromass apparatus. The anti-PPE activity of the desalted inhibitor was assayed as above.

Effect of pH and temperature on inhibitor activity

Greglin was incubated for 20 hours at 37°C in 0.5 M glycine-HCl buffer pH 2.5; 50mM hepes buffer pH 7.4; and in 0.5 M Tris–base pH 11.0, and its residual inhibitory activity assayed in the hepes buffer. The effect of temperature was investigated by incubating greglin (3.85 µM) in hepes buffer pH 7.4 for one hour at temperatures from 37°C to 90°C and then measuring its residual inhibitory activity at 37° C.

Reaction of greglin with PPE and HNE under acidic conditions

The greglin reactive bond (P1-P1') was tentatively identified by incubating the inhibitor with target enzymes at low pH and looking for putative cleavage sites by N-terminal sequencing, as described for soya-bean trypsin inhibitor [22]. Experimental details are provided in the "Supplementary Material" section.

Susceptibility of greglin activity to proteolytic enzymes

The susceptibility of greglin anti-elastase activity was investigated by incubating the inhibitor with serine (trypsin, endoprotease Glu-C), cysteine (papain) and metallo (pseudolysin) proteases. The mixtures were then separated by rp-h.p.l.c. and the inhibitory properties of the eluted peaks analysed (see Supplementary Materials).

RESULTS

Purification of two greglin isoforms from a crude homogenate of S gregaria ovaries:

We isolated two forms of an anti-PPE protein referred to as "greglin" from the *Schistocerca gregaria* ovaries using a combination of salt precipitation, size-exclusion and anion exchange chromatographies. The inhibitory activity of greglin was used to monitor its presence along the purification procedure. The protein was eluted from the Mono Q column as two close peaks that were further purified by rp-h.p.l.c on a Brownlee C4 column for sequence analysis, and gave a single band when run on a SDS gel (Figure 1A). A homogenate of 150 ovaries gave about 4 mg of purified greglin.

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Primary structure:

The sequence of each greglin isoform was obtained by sequencing overlapping peptides produced by cleaving the reduced alkylated rp-h.p.l.c.-purified proteins with cyanogen bromide (Met 14 and Met 68), trypsin (Lys 52, Lys 70 and Lys 81) and chymotrypsin (Leu 27, Tyr42 and Tyr 6) (Figure 1B). Both sequences contained 83 identical amino-acid residues except that one sequence had no identifiable residues at positions 8, 11 and 15, whereas the other had seryl residues at these positions. This strongly suggests that the seryl residues in the first sequence are glycosylated or phosphorylated. The molecular mass calculated from the greglin sequence was 9,229 Da, while mass spectrometry gave a minimal mass of 9,600 Da for and a maximal mass of 9,844 Da, consistent with a triphosphorylation. Intermediate masses consistent with mono and diphosphorylation were also recorded. The Net Phos program of the Expasy server predicted the phosphorylation of the Ser residues at positions 6, 8, 11 and 15 and 37 in agreement with sequence data that found no seryl residues at positions 8, 11 and 15 in one isoform, i.e. those with the best predictive scores. But there may well be additional post translational modifications that could explain the differences between the mass deduced from amino acid analysis and those obtained experimentally. We identified 6 Cys residues unambiguously by N-terminal sequencing after 4-vinyl-pyridine alkylation. The C-terminal sequence was confirmed by carboxypeptidase P digestion plus mass spectrometric analysis of the products. The data indicated a 303 Da decrease in the molecular mass, consistent with the KSS C-terminal sequence. In addition, cleavage of the native inhibitor with trypsin gave a molecule of lower mass (-175 Da), in agreement with the release of the two C-terminal seryl residues. Incubation of the trypsin-reacted products with carboxypeptidase P reduced the molecular mass by a further 128 Da, confirming the presence of a lysine residue at position 81.

We found no significant relationship between the whole sequence of greglin and any of the protease inhibitors searching the MEROPS database [23]. However using only part of the sequence (residues P26 to C69), the program retrieved two putative inhibitors from *Ciona intestinalis* (O97362) and from *Drosophila melanogaster* (Q9VE57) which share 38% and 36% homology, respectively, with this part of the greglin sequence.

We tentatively aligned the greglin sequence with those of these two peptides and with ovomucoid domain 3 (OMTKY-III), a typical Kazal inhibitor [24], and with the Kazal-related peptide PEC-60 [25], identified by fold recognition methods as the closest structural homologue to greglin (see

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next paragraph). The best scores were obtained with the *C intestinalis* and *D melanogaster* peptides; these were the only two that gave significant E-values (Table I). However, only three of six Cys residues in greglin aligned with those in the Kazal-related molecules (Figure 1C). This suggests a different disulfide bridge pattern, but we failed to identify this pattern due to the resistance of greglin to proteolysis. We then used computer-based structural studies to tentatively assign greglin to a family of peptidase inhibitors.

Secondary and tertiary structure predictions:

The secondary structure elements of the full-length greglin sequence (83 amino acids) were predicted using 7 different programs (Fig. 2A). All the programs except SABLE agreed on the presence of three main beta strands in greglin at positions 27-35, 41-45 and 59-64. They also predicted an alpha helix starting at R66 up to L76. The characteristic scaffold of the Kazal family of inhibitors consists of a central alpha helix, an adjacent three-stranded beta-sheet and a proteinase-binding loop cross-connected through disulfide bridges 1-5, 2-4 and 3-6, whereas inhibitors of the pacifastin family have beta strands only as secondary structure elements [26]. The predicted secondary structure of greglin is thus more closely related to that of Kazal inhibitors than to that of pacifastin inhibitors.

Because few sequence homologies were found by searching sequence databases (max 38%), we used protein fold recognition methods via the "GeneSilico" metaserver. The folding of greglin is predicted to contain alpha helices and beta sheets ("a+b" group of the SCOP classification; http://scop.mrc-lmb.cam.ac.uk/scop/). Most of the fold-recognition programs identified PEC-60 (PDB ID: 1PCE) as the closest structural homologue of greglin. Intermediate models (those not containing the variable loops) that correspond to the best hits of the fold recognition searches were aligned structurally to PEC-60 using the tool "Domain fit" of "SwissPdbViewer" (Fig 2B). The cores are readily superimposed, but there are deviations in the loops and the N-terminal region of 1PCE could only be aligned with one model (Figure 2C). We manually improved the structural alignment of greglin using insertions and deletions (Figure 2B). However, only one disulfide bridge was generated by this approach while there are 3 disulfide bridges in the folds of the Kazal-type inhibitors. Therefore, greglin appears to be only distantly related to the Kazal-type inhibitors and it could well have a novel folding pattern.

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Inhibition of serine proteases by greglin

The inhibitory activity of greglin was assayed toward a series of serine protease to delineate its specificity. In addition to PPE used to monitor its purification, those included the related human neutrophil proteases Cat G and protease 3, porcine trypin, bovine pancreatic chymotrypsin and subtilisin Carlsberg. Preliminary experiments showed that both greglin isoforms reacted similarly with the enzymes tested, so that both were equally used in the following kinetic experiments. Preliminary experiments also showed that HNE forms a much tighter complex with greglin than does PPE. We used this property to titrate the inhibitor. Increasing quantities of the inhibitor were reacted with 0.38 μM HNE as describe in the experimental section. The residual enzyme activity decreased linearly with the greglin concentration up to about 90 % inhibition, indicating that the inhibitor binds the enzyme tightly under the experimental conditions used (Figure 3), and suggesting a value of K_i at least two orders of magnitude lower than the HNE concentration used, in accordance with theory [20]. This experiment could not be used to determine K_i but allowed accurate titration of the inhibitor by fitting the data of the linear portion of the curve to the best theoretical straight line, whose intercept with the x axis was used to calculate the concentration of the active greglin (Figure 3A). Since the present titration data could not be used to measure K_i, the equilibrium dissociation constant for the greglin : HNE complex, this parameter was obtained from k_{ass} and k_{diss}, the association and dissociation rate constants. These parameters were measured using the progress curve method. Enzyme and inhibitor were reacted under pseudo-first order conditions ($[I]_0 \geq 10$ x $[E]_0$) in the presence of 0.2 mM MeOsuc-$(Ala)_2$-Pro-Ala-TBE with stopped flow mixing and detection. The rate of HNE inhibition was investigated at various inhibitor concentrations (Figure 3B).

The inset of Figure 3B shows a typical progress curve used to follow the time course of HNE inhibition. In all cases a pre-steady-state of product accumulation preceded its release at a constant rate, indicating a reversible interaction between enzyme and inhibitor. Non-linear regression analysis of the progress curves (Figure 3B) yielded k, the apparent pseudo-first order rate constant for the approach to equilibrium [20].

k increased linearly as a function of the inhibitor concentration (Fig 3B), suggesting that no reaction intermediate accumulates over the range of inhibitor concentrations used. Inhibition was therefore analysed assuming that HNE and greglin associate via a simple bimolecular

mechanism: the second order rate constant k_{ass} was calculated from the slope of the straight line on Figure 3B, its intercept with the ordinate yielding k_{diss}. We found k_{ass} = 1.23 ± 0.16 x 10^7 $M^{-1}.s^{-1}$ and k_{diss} = 0.043 ± 0.010 s^{-1}. K_i = 3.49 ± 1.25 x 10^{-9} M was deduced from $K_i = k_{diss}/k_{ass}$..

The K_i for the complexes between greglin and the other enzymes were obtained from equilibrium titration experiments with experimental conditions compatible with concave inhibition curves. The effects of increasing greglin concentrations on PPE activity (18 nM final) are illustrated in Figure 4. The residual enzyme activity was assessed by adding the substrate (Suc-$(Ala)_3$-*p*-NA) to the equilibrium reaction mixtures. The rate of product release accelerated for about 30 s and reached a steady state. This time course of substrate breakdown indicates three things: first, that the enzyme partially dissociates from a reversible complex with inhibitor as a result of disturbance by substrate of the initial equilibrium; second, that substrate and inhibitor compete for binding to PPE; and third, that a new equilibrium between PPE, greglin, substrate and their complexes is reached. The plot of the steady state rate of substrate hydrolysis *versus* the inhibitor concentration was analyzed [16] to obtain $K_{i(app)}$, the substrate-dependant equilibrium dissociation constant for the greglin : PPE complex (Figure 4). The true K_i value was obtained from $K_i = K_{i(app)}/(1+[S]_0/K_m)$, where $[S]_0$ is the initial substrate concentration and K_m the Michaelis constant obtained by classical means.

This procedure was used to study the interaction of greglin with bovine chymotrypsin, subtilisin Carlsberg and the human cathepsin G. The K_i for all enzyme : inhibitor pairs are shown in Table II. Porcine trypsin and protease 3 were not inhibited under these conditions.

Greglin physicochemical properties

Resistance to proteolysis: Greglin was incubated with HNE and PPE at low pH to locate the enzyme-binding site through a peptide bond cleavage [22]. Sequence analysis of the rp-h.p.l.c.-fractionated mixtures revealed that greglin had been N-terminally truncated at E10, Q12 and L27 by PPE and at M14 by HNE. The N-terminally cleaved inhibitor (minus 26 residues) retained full inhibitory activity towards PPE or HNE at pH 7.4. The observation that P1-P26 N-terminal segment is not involved in inhibition agrees with the total lack of homology between this segment and the sequences of other protease inhibitors.

Native greglin was also incubated with other proteases, including serine (trypsin and Glu-C), cysteine (papain) and metallo (pseudolysin) proteases, at various molar ratio. The resulting

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mixtures were fractionated by rp-h.p.l.c. and the main peaks sequenced and analysed for their HNE-inhibitory properties. Again the cleavage sites were located within the N-terminal segment (P1-P26), except that trypsin cleaved at K81 before the penultimate C-terminal residue of the protein. Greglin remained a potent HNE inhibitor even after prolonged incubation and fractionation by rp-h.p.l.c., whatever the protease used. We conclude that N-terminal (minus 26) and C-terminal (minus 3) truncations of greglin do not alter its inhibitory properties.

The resistance of greglin anti-HNE activity to pseudolysin was also compared to that of α_1-P_i, the major physiological inhibitor of HNE. Figure 6A shows the effect of incubating greglin and α_1-PI with various amounts of pseudolysin for 15 min on their anti-HNE activity. Greglin retained most of its inhibitory capacity even at the highest enzyme concentration used, whereas α_1-Pi activity was reduced by 80 % at E = 50 nM or completely lost at E = 500 nM (Figure 5A).

pH and temperature stability: The recovery of inhibitory activity after rp-h.p.l.c. demonstrated that the two forms of greglin resist denaturation by acid and acetonitrile. We investigated the resistance of the inhibitor to low and high pH by incubating it overnight at pH 2.5 and at pH 11.0. The inhibitory properties were not significantly altered (Figure 5B). Similarly, we found no significant loss of activity when the inhibitor was incubated in hepes buffer for one hour at temperatures from 37 to 90°C (data not shown).

Sensitivity to oxidation and reduction : Chemical and physiological oxidizing system can alter the biological activity of many proteins by converting their exposed methionyl residues to methionine sulfoxide. This can dramatically lower the activity of protein protease inhibitors, especially those having a methionyl residue at position P1 or P1' of their reactive site [27-29]. Since greglin contains 3 methionyl residues, its activity might be altered by oxidants. The inhibitor was incubated with N-chlorosuccinimide and its oxidation checked by mass spectrometry. The observed molecular mass was consistent with the oxidation of the three Met residues but HNE-inhibitory capacity of oxidized greglin was unaltered (Figure 5C). This absence of significant change in the greglin anti-HNE property suggests that no methionyl residue occupies a critical position in the inhibitor reactive site. But greglin lost all its inhibitory capacity once its disulfide bridges had been reduced by 10mM dithiothreitol and alkylated with iodoacetamide. The greglin tertiary structure therefore is essential for inhibition, which suggests that the sequence from the first to the last Cys residues (Cys33 to Cys76) is required for inhibitory activity.

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Iinhibition of HNE in cystic fibrosis sputum: Purulent sputum is a complex biological medium containing elements (oxidants, proteolytic enzymes) able to depress the activity of local physiological inhibitors [30]. We reacted increasing amounts of greglin with fixed amounts of cystic fibrosis sputum supernatant to delineate the anti-HNE efficiency of the inhibitor in sputum. The remaining HNE-activity was measured by adding the fluorescent substrate Abz-APEEIMRRQ-EDDnp to the equilibrium mixtures. The relative enzyme activity decreased curvilinearly with the quantity of inhibitor (Figure 6), as anticipated from the K_i value in hepes buffer (Table II), the greglin concentration, and the protease concentration (30nM) that gave a [HNE]/*Ki* ≈ 8.5 [16]. Since inhibition of HNE by greglin in sputum is best described by the K_i measured in this environment, we analyzed the inhibition data as indicated in the legend of figure 4 to determine this parameter. The concave inhibition curve shown in figure 6 was generated using the best estimate of $K_{i(app)}$. After correction for the substrate (K_m = 15 μM, determined by classical methods), we found K_i = 3.1 ± 0.43 nM, a value close to that determined by stopped-flow kinetics with the purified enzyme:inhibitor system. Thus the sputum components did not inactivate the anti-HNE function of greglin during the time of the experiment and did not dissociate the enzyme-inhibitor complex.

DISCUSSION

Anti inflammatory therapy using protease inhibitors has long been suggested as an efficient means of controlling the unopposed proteolytic activity that occurs at inflammatory sites. Neutrophil elastase is one of the main proteases released from activated and dying PMNs and it is involved in tissue destruction and inflammation. A variety of natural, recombinant and synthetic inhibitors have been used as anti-inflammatory drugs to target HNE but the results to date have not been entirely satisfactory [1]. New elastase inhibitors that have different structural and/or physicochemical properties from those described so far could lead to new, therapeutically useful elastase inhibitors.

Arthropods and other invertebrates are a rich source of protease inhibitors. Several belonging to the Kunitz and serpin families have been isolated, especially from haemolymph [31]. Two new

families of low molecular weight serine protease inhibitors were discovered recently: one in silkworms [32] and the other in locusts [6, 7, 17 , 33] and crayfish [34]. The function of these serine protease inhibitors and their distribution in invertebrate, especially arthropods, remains a matter of debate; but this could be due to the far larger number of serine proteases in insects than in human and other vertebrates [4]. Five serine protease inhibitors, designated SGPI 1-5, have been isolated from the ovaries of the locust (*S. gregaria)* [7]; they are members of the pacifastin family of peptidase inhibitors. These low Mr (about 35 residues) inhibitors are found only in invertebrates and they all have a similar cysteine array [5]. Aqueous homogenates of mature locust ovaries also contain potent trypsin, chymotrypsin and elastase inhibitory activities. The trypsin and chymotrypsin inhibitor is a 14 kDa, heat stable peptide that has been purified and characterized [8], but the protein(s) responsible for the anti-elastase activity is still unidentified.

The protease inhibitors we have purified from the locust ovary are two forms of a single protein, greglin. They have similar inhibitory behaviors towards all the tested proteases. Greglin reversibly inhibits enzymes of the elastase and chymotrypsin families. It forms tight complexes with subtilisin and HNE, but has no effect on the activities of trypsin or protease 3. To our knowledge, no other potent HNE inhibitor has been purified from locusts. Most of the known locust inhibitors belong to the pacifastin family and preferentially inhibit trypsin and chymotrypsin [8, 17]. The sequence of greglin is only distantly related to that of any other protease inhibitor. The putative Kazal-type inhibitor of *Ciona intestinalis* is the most closely related, as the C-terminal region of the sequence is 38% homologous. The function of the greglin N-terminal extension that takes no part in inhibition remains unexplained, but its sensitivity to proteolysis contrasts with that of the inhibitory C-terminal domain. Though this domain has some physicochemical properties in common with members of other low Mr inhibitors, it also has features that can be exploited in drug development.

The stopped–flow kinetics of HNE inhibition by greglin show that the latter is a fast acting inhibitor that binds the enzyme *via* a simple bimolecular mechanism under the experimental conditions used. The HNE : greglin complex formation is governed by the second order rate constant k_{ass} = 1.23 x 10^7 $M^{-1}s^{-1}$, a value that is similar to, or higher than that reported for the main physiological inhibitors of this enzyme: α_1-PI (1.3 x 10^7 $M^{-1}s^{-1}$ [35], which irreversibly inhibits its target proteases, and the reversible inhibitors SLPI (6.4 x 10^6 $M^{-1}s^{-1}$ [36]) and elafin

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(3.6×10^6 $M^{-1}s^{-1}$ [37]). The rate of HNE inhibition by greglin is also similar to that measured for eglin c ($k_{ass} = 1.3 \times 10^7$ $M^{-1}s^{-1}$ [38]), another elastase inhibitor from the leech. The K_i for the HNE : greglin complex is significantly higher than those for the interactions between HNE and the reversible inhibitors SLPI (0.3 nM [36] and elafin (0.2nM [37]). Despite this relatively low affinity, greglin concentrations of about 10^{-8} molar efficiently inhibit the elastase activity in purulent sputum.

Greglin also resists oxidation, while α_1-PI, SLPI and elafin, the major protease inhibitors of the lung, do not. The HNE-inhibitory activity of these three human proteins is reduced by their interaction with the oxidant species produced by the myeloperoxidase – hydrogen peroxide system of triggered neutrophils [39], or with N-chlorosuccinimide [27, 28]. Oxidative inactivation of the inhibitors is due to the conversion of a methionyl residue involved in protease binding to methionine sulfoxide. As greglin is not inactivated by incubation with N-chlorosuccinimide, it probably has no methionyl residue in such a critical position (P1 or P'1) in its reactive site. This also suggests that greglin would retain its anti-elastase activity in a chronic inflammation environment with high concentrations of oxidant species and lysosomal proteinases produced by continuously recruited neutrophils.

Pseudolysin, a metalloenzyme that contributes to the virulence of *P aeruginosa*, can break down many proteins, including α_1-PI and secretory leukocyte protease inhibitor. Both inhibitors are inactivated by limited cleavage in their reactive site regions [40, 41]. This mechanism, in which inhibitors behave as substrates, may cause a significant loss of active inhibitors and promote the breakdown of lung connective tissues by neutrophil proteases. We find that greglin resists inactivation by several proteolytic enzymes, including high concentrations of trypsin, papain and pseudolysin. This is important for the development of a therapeutic system, since *P aeruginosa* infection accompanies the inflammation of airways in patients with cystic fibrosis [42].

The predicted secondary and tertiary structures suggest that greglin is more closely related to the Kazal inhibitors than to pacifastin inhibitors. However the distribution of Cys residues within the greglin sequence does not fit the disulfide pattern found in classical Kazal inhibitors, which all contain three disulfide bridges. Though the inhibitory site of Kazal inhibitors lies within the Cys2-Cys4 disulfide loop, computer-based analysis predicts that the greglin inhibitory site lies in the N-terminal extended strand, part of which is not necessary for inhibition. We therefore believe that greglin is folded in a manner different from that of classical Kazal-type

inhibitors. However, non-classical Kazal-type inhibitors with different folding [43] or different positioning of their cysteine residues [44] have been described. Our results and observations suggest that it is difficult to assign a fold to the greglin sequence using computer-based methods alone. For example, none of the *ab initio* models constructed by the Robetta server (http://robetta.bakerlab.org/) was folded like any known inhibitor (not shown).

The resolution of the greglin 3D structure or the production of recombinant forms with single mutation points at the putative inhibitory site will be needed before we can assign this inhibitor to a family and understand the molecular basis of its physicochemical properties.

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Figure Legends

Figure 1: Purification of greglin: (A) SDS gel electrophoresis of purified greglin after ion exchange chromatography (B) Complete amino-acid sequence of greglin obtained from N-terminal sequencing of overlapping peptides produced by chemical and proteolytic cleavages followed by rp-h.p.l.c separation of each greglin isoform after reduction and alkylation. Proteolytically-cleaved and chemically-cleaved fragments are underlined.
(C)Alignment of the sequences of greglin (gre) and kazal-related homologues calculated using T-coffee (*http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi*)[45]. (OMTKY-III (turkey ovomucoid domain 3) (ovo), porcine PEC-60 (pec), *C intestinalis* (cio) and *D melanogaster* (dro)). The inhibitory site of OMTKY-III (P4 to P3') is underlined. The scores resulting from the the multiple sequence analysis are identified as bad, average and good.

Figure 2: Prediction of the greglin secondary and tertiary structures :
(A) The full amino acid sequence of greglin is displayed on the first line. Each of the other 7 lines reports the secondary structure predicted by the different programs used. The residues predicted to be involved in beta-strands are annotated with the letter "E ". The residues that are predicted to be in helical regions are annotated with the letter "H".
(B) Fold recognition sequences and fold recognition structural alignments. Sequence alignments were based on the structural alignment between 1PCE and a subset of "intermediate" models generated by the Genesilico server from the best hits of the fold recognition search. The first line is the greglin sequence, which was fitted manually to the template structure (1PCE). The identifier at the beginning of each line refers to the program (ffas, inbgu, sam) and the structure (1PCE, 1BUS, 1OVO, 1ROT) used to build each of the "intermediate" models. SwissPdbViewer (Domain Fit) was used to calculate the structural superimposition and generate the corresponding alignment. Residues in light grey are the residues of the intermediate models that were aligned with those of the template 1PCE by the fitting operations.

(C) Superimposition of PEC-60 (1PCE, black) and the 6 best intermediate models for the greglin sequence. Only the carbon-alpha (traces) of the polypeptides are displayed. The colors used are as follows: ffas-1HPT, yellow; inbgu-1BUS, green; ingbu-1OVO, purple; sam-1ROT, blue. The structures corresponding to ffas-1PCE and ingbu-1PCE can be superimposed on those represented, and are not visible. The secondary structures of the template 1PCE are identified by the letter "H" for the alpha helix and "β" for the beta-strands.

Figure 3:

(A) Effect of increasing quantities of greglin on the activity of 0.38 μM HNE. The enzyme relative activity is the ratio of the steady state rate of Suc-$(Ala)_3$-*p*-NA hydrolysis (see text) measured in the presence of inhibitor to the rate measured in its absence. The straight line drawn through the data is theoretical. It shows that greglin titrates the enzyme under the experimental conditions used. Its intercept with the x axis was used to calculate the concentration of the active inhibitor.

(B) Linear dependence of k, the pseudo-first order rate constant for HNE inhibition as a function of the greglin concentration, indicating that I and E interact according to a bimolecular mechanism. k_{ass} and k_{diss} could therefore be obtained from a linear fit of the data to equation 1 [16].

$$k = k_{diss} + k_{ass}[I]_0 / (1 + [S]_0 / K_m) \qquad (1)$$

The theoretical straight line shown was generated using the best estimate of k_{ass} and k_{diss} (1.23 ± 0.15 x 10^7 $M^{-1}.s^{-1}$ and 4.30 ± 0.97 x 10^{-2} s^{-1}, respectively) with K_m = 20.9 μM for the MeOsuc-$(Ala)_2$-Pro-Ala-TBE : HNE interaction.

Inset: stopped-flow trace showing product accumulation as a function of time for the reaction of 0.32 μM greglin with 30 nM HNE in the presence of 0.2 mM MeOsuc-$(Ala)_2$-Pro-Ala-TBE. The best estimate of the rate constant k was obtained, as published [16], by fitting the progress curve data to equation 2:

$$[P] = v_s t + (v_z - v_s)/k(1 - e^{-kt}) \qquad (2)$$

where [P] stands for the product concentration at any time t and v_z and v_s are the velocity at t = 0 and the steady state velocity, respectively. The best theoretical curve represented by a smooth line was generated with k = 0.45 ± 0.035 s^{-1}, v_z = 2.98 ± 0.14 μM.s^{-1} and v_s = 0.29 ± 0.013 μM.s^{-1}.

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Figure 4:

Effect of increasing quantities of greglin on the activity of 18 nM PPE. The residual enzyme activity was measured with (Suc-$(Ala)_3$-*p*-NA) as indicated in the text. $K_{i(app),}$ the substrate-dependant K_i, was obtained by fitting the experimental equilibrium titration data to equation 3 [16]:

$$a = 1 - \left[\left([E]_0 + [I]_0 + K_{i(app)} \right) - \sqrt{\left([E]_0 + [I]_0 + K_{i(app)} \right)^2 - 4[E]_0[I]_0} \right] \Big/ 2[E]_0 \qquad (3)$$

where *a* is the enzyme relative activity as defined in the legend of figure 3. $[E]_0$ and $[I]_0$ are the initial enzyme and inhibitor concentration, respectively. The theoretical curve was generated with $K_{i(app)}$ = 0.102 µM.

Figure 5 :

(A) Effect of pseudolysin on the anti-HNE activities of greglin and α_1-Pi : Inhibitors (4µM final) were incubated with pseudolysin (0.5 nM-0.5 µM) as indicated in the text. Results show the median and maximal values ($n \geq 2$)

(B) Resistance of greglin activity to oxidation and acetonitrile: Greglin 2 (77 µM final) was incubated with 125 µM N-chlorosuccinimide. Residual greglin inhibitory activity was then measured and compared with those of the untreated inhibitor and inhibitor after rp-h.p.l.c. The HNE concentration was adjusted so that inhibition by native greglin was incomplete (90%). Results show the median and maximal values ($n \geq 2$)

(C) Resistance of greglin activity to acid and base conditions: Solutions of purified greglin were incubated at pH 2.5, pH 7.4 and pH 11.0 at 37°C overnight and their capacity to inhibit neutrophil elastase measured. Results show the median and maximal values ($n \geq 2$)

Figure 6:

Inhibition of sputum elastase activity by greglin. Aliquots of homogenized sputum containing a constant concentration of HNE were reacted with increasing quantities of greglin. The final enzyme concentration (30nM) was estimated as described in the text and Abz-APEEIMRRQ-EDDnp was used as the substrate. Under the experimental conditions used, the decrease of HNE activity *vs* the inhibitor concentration is concave, therefore the data could be analyzed as

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described in the legend of figure 4 to obtain $K_{i(app)}$. The curve was generated using equation 3 and $K_{i(app)}$ = 3.28 nM.

Table I :

Sequence alignments of greglin residues P26 to C 69 with Kazal-related inhibitors from Ciona intestinalis (Swiss-Prot ID: 097362), Drosophila melanogaster (Swiss-Prot ID: Q9VE57), pig PEC-60 (Swiss-Prot ID: P37109) and OMTKY3 (Swiss-Prot ID: P68390) (using the LALIGN program from Fasta.E value : homologous proteins were taken to be those for which the aligned amino acid sequences were related with an E value of 0.001 or less*

sequence comparison	% alignment	E Value
greglin-O97362	36	0.0005
greglin-Q9VE57	33	0.00091
O97362-Q9VE57	27	0.014
greglin-P37109	34	1.7
Greglin-P68390	27	1.400

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Table II:

Equilibrium dissociation constant (K_i) for the interaction of greglin with the various proteases.

Enzyme	K_i (nM)
PPE	58.3 ± 12
HNE	3.6 ± 2.1[a]
PR 3	NI[b]
cathepsin G	153.5 ± 10
chymotrypsin	26.7 ± 10.8
subtilisin	0.68 ± 0.10
trypsin	NI[b]

[a]calculated from k_{ass} and k_{diss} (see text)

[b]NI, no inhibition

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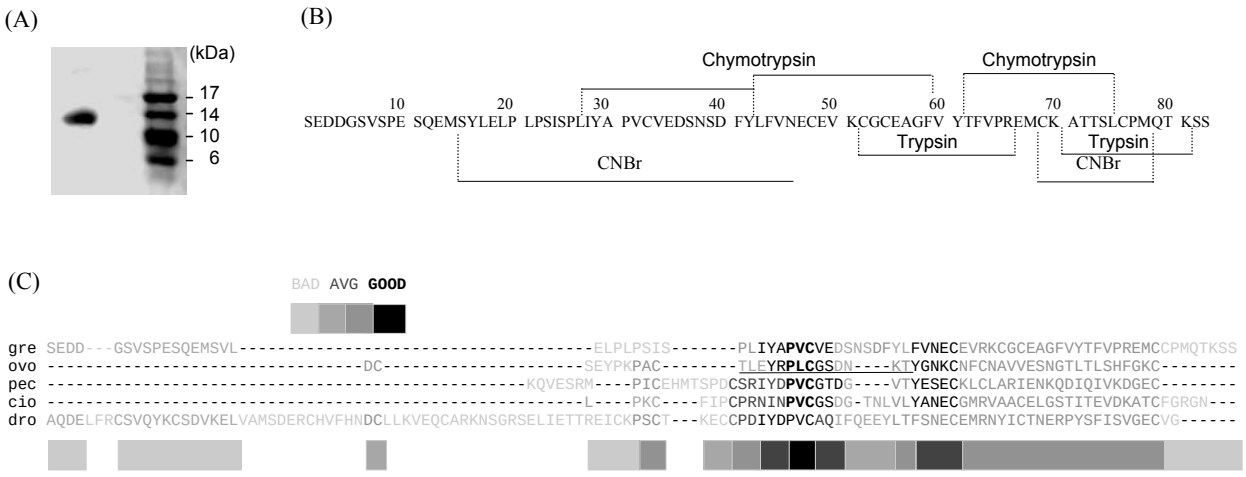

Brillard-Bourdet et al : Figure 1

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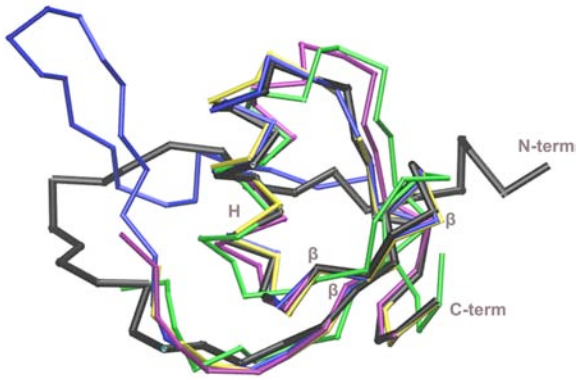
(A)

```
1   .   10     .  20     .  30     .  40    .   50    .   60    .   70    .   80
SEDDGSVSPESQEMSYLELPLPSISPLIYAPVCVEDSNSDFYLEVNECEVRKCGCEAGFVYTFVPREMCKATTSLCPMQTKSS
--------------EEEE--------EEEEEEEEE-----EEEEE---EEEE-------EEEE--HHHHHHHHHH--------    PSIPRED (1)
----------H-----E----------EEEEEEEE-----EEEEE--E---------EEEEE-HHHH--------------    SAM (2)
----------------HH---------EEEEEEEE-----EEEE-----HHH------EEEEEE-HHHHH-------------    JUFO (3)
--------------EE----------EEE--EEEE-----EEEEE---EE--------EEEEE--HHHHHHH-----------    SSPRO (4)
---------------------------------E--------EEEE---------------------HHHHH-------------    SABLE (5)
-------------EEEEE---------EE--EEEE-----EEEEEEEEEEE------EEEEE--HHHHH-------------    PROFSEC (6)
---------------H----------E-------------EEE--------------EEEE----HHHH--------------    NNPREDICT (7)
```

(B)

```
Greglin  SEDDGSVSPESQEM--SYLELPLPSISPLIYAP-VCVEDSNSDFYLFVNECEVRKCGCEAGF--VYTFVPREMCKATTSLCPMQT
1PCE     EKQVFSRMPICEHM-------TESPDCSRIYDP-VCGTDG--VTYE--SECKLCLARIENKQ--DIQIVKDGEC
ffas-1HPT                          SPLIYAP-VCVEDS--NSFV--NECEVRKCGCEAGF--VYTFVPREMC
ffas-1PCE                              YAP-VCVEDS--NSFV--NECEVRKCGCEAGF--VYTFVPREMC
inbgu-1BUS                       PISPLIYAP-VCDSNS--DFYL--NECEVRKCGCEAGF--VYTFVPREMC
ingbu-1OVO                        ISPLIYAP-VCDSNS--DFYL--NECEVRKCG--CEAGFVYTFVPREMC
ingbu-1PCE DDGSVSPESQEM-------PLPSISPLIYAP-VCDSNS--DFYL--NECEVRKCGCEAGF--VYTFVPREMC
sam-1ROT       EDDGSVSPESQEMSLPLPSISPLIYAP-VCNSDF--YLEV--NECEVRKCGCEAGF--VYTFV
```

(C)



Brillard-Bourdet et al : Figure 2

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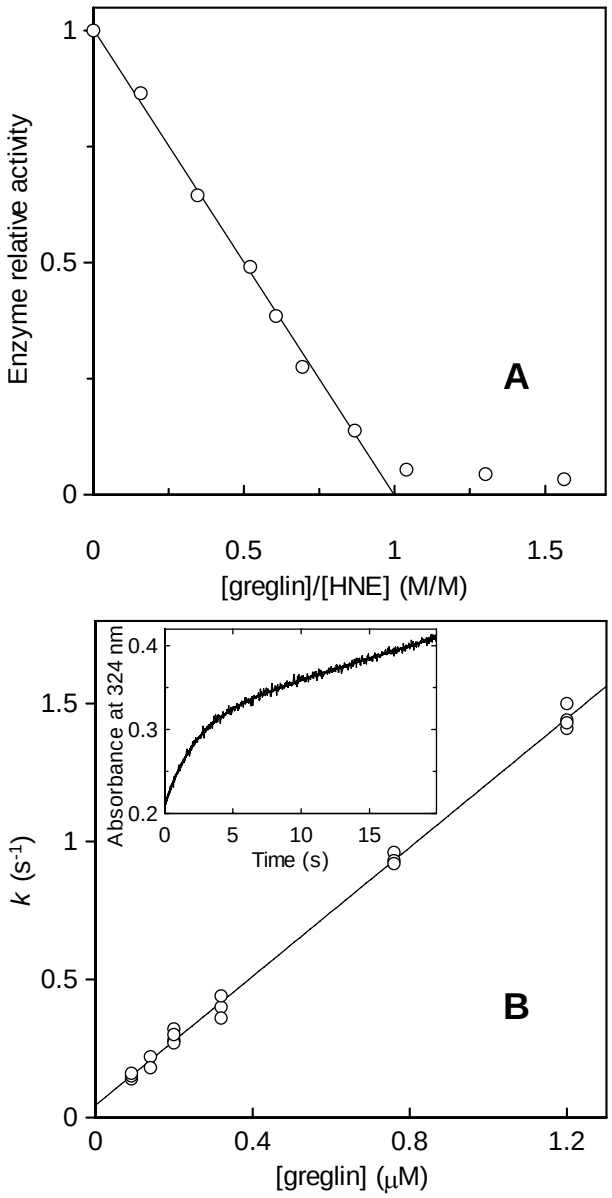

Brillard-Bourdet et al: figure 3

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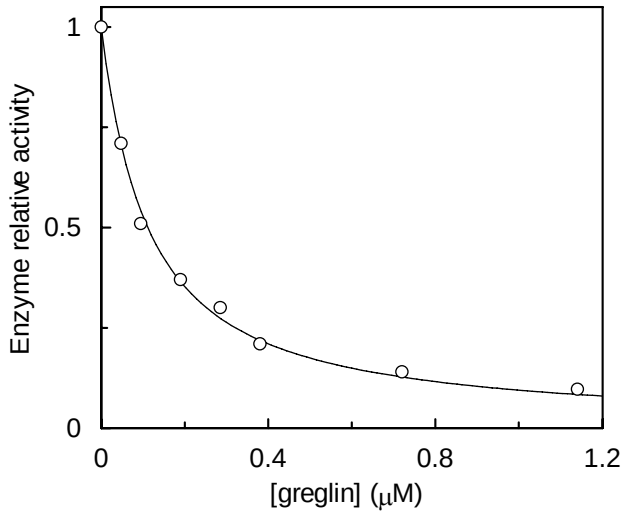

Brillard-Bourdet et al: figure 4

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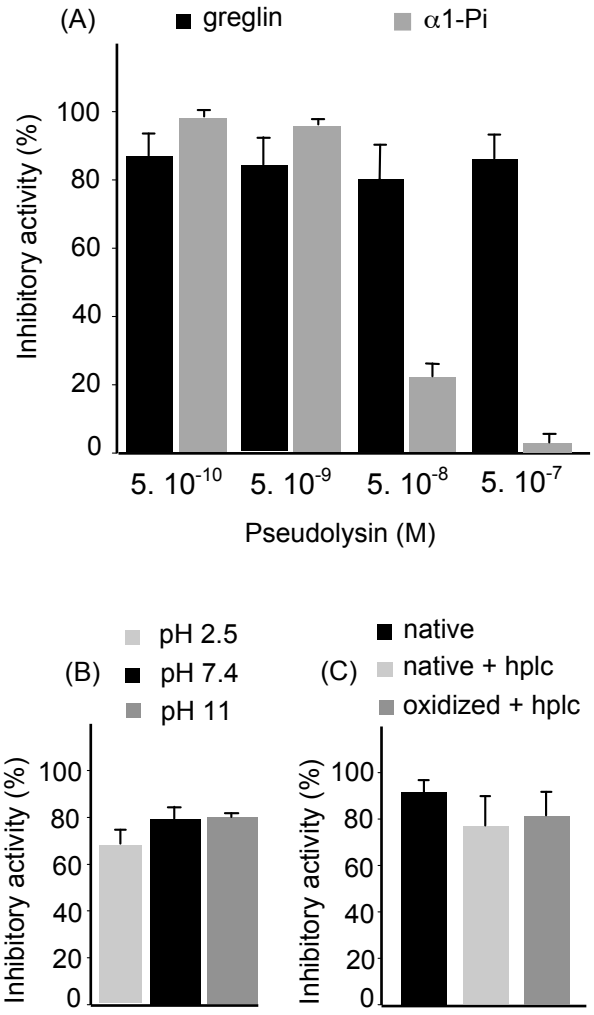

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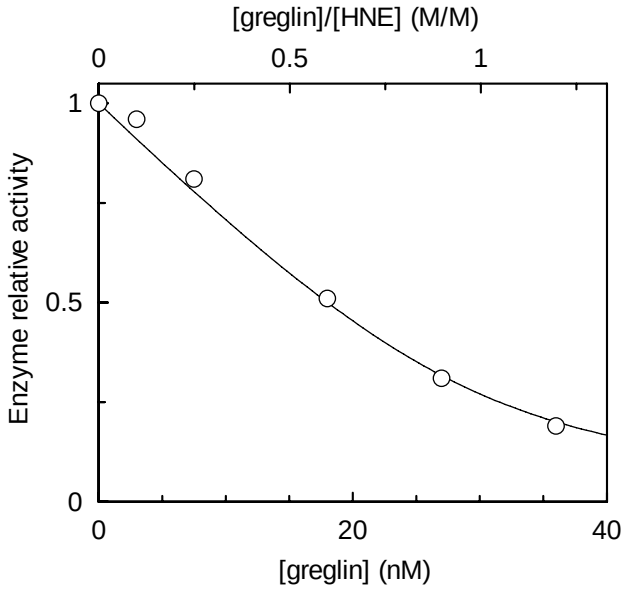

Brillard-Bourdet et al: figure 6