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INACTIVITY OF RECOMBINANT ELA2B PROVIDES A NEW EXAMPLE OF EVOLUTIONARY ELASTASE SILENCING IN HUMANS

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Abstract

BACKGROUND. The archetypal mammalian elastase (ELA1) is not expressed in the human pancreas, because evolutionary mutations suppressed transcription of the *ELA1* gene. *AIMS*. In this study we tested the theory that the unique duplication of the *ELA2* gene in humans might compensate for the loss of ELA1. *METHODS*. Recombinant ELA2A and ELA2B were expressed in *Escherichia coli*, and their activity was tested on Glt-Ala-Ala-Pro-Leu-p-nitroanilide, DQ elastin and bovine milk protein. *RESULTS*. Surprisingly, recombinant ELA2B was completely devoid of proteolytic activity, while ELA2A readily hydrolyzed all three test substrates. Furthermore, ELA2A formed an SDS-resistant complex with α1-antitrypsin, whereas ELA2B did not bind covalently to the inhibitor. Finally, chimeras and point-mutations engineered between ELA2A and ELA2B revealed that multiple evolutionary mutations inactivated ELA2B. *CONCLUSIONS*. The results indicate that ELA2B is not an elastase enzyme and confirm that ELA2A is the major elastase in the human pancreas.

Keywords

pancreatic elastase; gene duplication; pseudogene; antitrypsin

INTRODUCTION

Elastases are defined by their ability to release soluble peptides from insoluble elastin fibers by a proteolytic process called elastinolysis or elastolysis. The common pancreatic elastase (ELA1) routinely purified from pig or cow pancreas is not expressed in humans due to transcriptional silencing [1-3]. For unclear evolutionary reasons, the human *ELA1* gene has accumulated mutations in the 5′ upstream region which inactivated both its promoter and enhancer [3]. Interestingly, the *ELA1* coding region has remained intact, suggesting that ELA1 might be expressed in other tissues. Indeed, ELA1 expression was demonstrated in human skin cells, and a frequent deletion polymorphism was also identified [4]. In humans, elastase 2 is the only digestive enzyme with reported elastinolytic activity in the pancreas [5,6]. Cloning of the ELA2 cDNA yielded two highly similar sequences, of which ELA2A corresponded to the enzyme previously purified from human pancreas, whereas ELA2B coded for a novel elastase with ~90 % identity to ELA2A [7-9]. Genomic sequencing confirmed that ELA2A and ELA2B are products of two apparently duplicated *ELA2* genes. The *ELA2A* and *ELA2B* genes are

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approximately 15 kB long and are located only ~4 kB apart on chromosome 1. The expression of both isoforms at the mRNA level has been also confirmed by numerous EST-sequences.

We hypothesized that duplication of the *ELA2* gene might be a compensatory mechanism for the loss of ELA1. To test this notion, we have expressed ELA2A and ELA2B recombinantly. Surprisingly, ELA2B proved to be another example of a "silent" human elastase with no detectable proteolytic activity.

METHODS

Materials. Elastase substrate Glt-Ala-Ala-Pro-Leu-p-nitroanilide was from Peptides International (Louisville, Kentucky) and DQ elastin was from Molecular Probes (Eugene, Oregon). Recombinant human anionic and cationic trypsins were obtained as described previously [10-12]. IMAGE clones were purchased from American Type Culture Collection (Manassas, Virginia).

Expression plasmids and mutagenesis. The cDNA for proelastase 2A and 2B was PCR-amplified from IMAGE clones #6226278 (GenBank accession CA952548) and #6124893 (GenBank accession BU784962), respectively, and cloned under the control of the T7 promoter in the pTrap-T7 expression plasmid using *Nco* I and *Sal* I restriction sites. For cytoplasmic expression in the *Escherichia coli* Rosetta (DE3) strain, the native elastase signal peptide (amino-acids 1-16) was replaced with a Met-Ala sequence. The forward primer carrying an *Nco* I site for ELA2A was 5′-GCT GGA ACC ATG GCT TGT GGG GAC CCC ACT TAC CCA CCT TAT GTG-3′; the forward primer for ELA2B was 5′-TCC CAC ACC ATG GCT TGT GGG GTC TCC ACT TAC GCG CCT GAT ATG-3; and the reverse primer carrying a *Sal* I restriction site for ELA2A and ELA2B was 5′-GAC TTC GTC GAC TTA GTT ATT TGC AAT CAC CGA ATT GAT CC-3′. Chimeras and point-mutations were generated by PCR-mutagenesis.

Sequence variations in ELA2B. We have noticed differences between the originally reported cDNA sequence (GenBank accession Ml6653) and the EST-clone we have purchased and sequenced. Specifically, the cDNA codons for Arg79 (AGG); Asn114 (AAC), and Thr158 (ACA) were found to be Gly79 (GGG) Asp114 (GAC) and Thr158 (ACG). A database search confirmed that all reported EST sequences were identical to the clone we obtained. Furthermore, the genomic sequence for ELA2B (GenBank accession NT_004873; *Homo sapiens* chromosome 1 genomic contig) also agreed with the EST clone at these positions. On the other hand, the ELA2B genomic sequence reports codon 177 as Gln (CAG), while both the cDNA and EST sequences confirm an Arg177 (CGG) codon at this position. Although sequencing errors may account for these discrepancies, the single-nucleotide polymorphism (SNP) database lists G79R (rs3820071), D114N (rs3766160), T158 (rs10927792) and Q177R (rs6429745) as ELA2B sequence variations, indicating that these differences probably represent true allelic variants.

Expression and purification of recombinant human pancreatic proelastase 2. The protocol previously developed for the expression, *in vitro* refolding and ecotin-affinity purification of human trypsinogens was used to obtain pure recombinant proelastase preparations [10-12]. Concentrations of proelastase solutions were calculated from their ultraviolet absorbance at 280 nm using a theoretical extinction coefficient of 72,860 $M^{-1}cm^{-1}$. Typical proelastase yields were ~50 μg of purified zymogen per 100 mL culture.

Elastase activity assays. Three test substrates were used to characterize the enzymatic activity of recombinant elastases. The small peptide substrate Glt-Ala-Ala-Pro-Leu-p-nitroanilide was described as the best turnover substrate for human ELA2A [13]. In our assays, the elastase-

mediated release of the yellow p-nitroaniline was followed at 405 nm using a Spectramax Plus 384 microplate reader (Molecular Devices). We have determined the catalytic parameters of recombinant ELA2A on Glt-Ala-Ala-Pro-Leu-p-nitroanilide (K_M 0.9 mM; k_{cat} 1.2 s^{-1}), and compared those to the published numbers obtained with purified native elastase 2A and Suc-Ala-Ala-Pro-Leu-p-nitroanilide (K_M 1.4 mM; k_{cat} 5.1 s^{-1}) [13]. The K_M values were comparable, while the k_{cat} of the recombinant preparation was somewhat reduced. DQ elastin is a fluorescent substrate supplied in the EnzChek® Elastase Assay Kit (E-12056) by Molecular Probes. DQ elastin is soluble bovine neck ligament elastin that has been labeled with the BODIPY FL fluorescent dye in a manner that the conjugate's fluorescence is quenched. The non-fluorescent substrate can be digested by elastase or other proteases to yield highly fluorescent fragments. We have used a SpectraMax Gemini XS fluorescent microplate reader (Molecular Devices) to measure the fluorescence change in our elastase assays using excitation and emission wavelengths of 485 nm and 538 nm, respectively. Finally, digestion of bovine milk proteins was used as a test of general proteolytic activity. Experimental details are given in the legends to the figures.

RESULTS

Recombinant expression of human proelastase 2A and 2B in *Escherichia coli*. The cDNA sequence for ELA2A and ELA2B were PCR-amplified and cloned into the pTrap-T7 expression plasmid that was developed for the expression of human trypsinogens (see *Experimental Procedures*). Proelastase was expressed as cytoplasmic inclusion bodies and subjected to *in vitro* refolding and ecotin-affinity purification.

Activity of ELA2A and ELA2B. Recombinant proelastase 2A and 2B were activated with human anionic or cationic trypsin for 10 min. Removal of the activation peptide by trypsin results in a small mobility shift on reducing SDS-polyacrylamide gels, which allowed us to confirm that trypsin-mediated activation was complete for both ELA2A and ELA2B (not shown). First, elastase activity was tested on the flurorescent DQ elastin substrate (Molecular Probes). Surprisingly, ELA2B exhibited no measurable activity on this substrate, while ELA2A generated a steadily increasing fluorescent signal as a function of time (Fig 1A). In control experiments, trypsin did not hydrolyze DQ elastin, when tested at the concentration used for proelastase activation (not shown). To characterize proelastase activation in more detail, ELA2A and ELA2B were activated with human trypsins and elastase activity was determined at different activation times with the synthetic chromogenic substrate Glt-Ala-Ala-Pro-Leu-p-nitroanilide (Fig 1B) or DQ elastin (not shown). In agreement with the first experiment, ELA2B has remained completely inactive, while ELA2A was rapidly activated by trypsin (Fig 1B).

An unexpected observation in the activation experiments was that human cationic trypsin was a much less efficient activator of ELA2A than anionic trypsin. The difference in rates of activation was approximately 10-20-fold under the experimental conditions in Fig 1. Interestingly, ELA2A activation by cationic trypsin showed an initial “burst” phase followed by slower “steady state” activation. This suggests that dissociation of the activated ELA2A is the rate limiting step in the reaction, which results in an apparent inhibition of cationic trypsin-mediated proelastase activation. Importantly, maximal elastase activities were achieved by activation with either human trypsin, indicating that trypsin did not degrade proelastase.

ELA2A was also activated by the lysosomal cysteine-protease cathepsin B at pH 4.0, although levels of elastase activity reached only about 20-30 % of maximal activity (not shown). On the other hand, cathepsin-B mediated activation of ELA2B did not result in any elastase activity whatsoever.

Finally, a crude bovine milk protein solution was digested with activated ELA2A or ELA2B and analyzed by SDS-PAGE. As shown in Fig 2A, ELA2A completely degraded milk proteins to peptides smaller than 15 kDa, while ELA2B was inactive.

Complex formation with α1-antitrypsin. Further evidence for the lack of catalytic potential of ELA2B was obtained by testing elastase binding to α1-antitrypsin. Alpha1-antitrypsin inhibits target serine proteases by forming a covalent acyl-enzyme intermediate through the catalytic Ser residue with the concomitant cleavage of the Met358-Ser359 peptide bond in the reactive center loop of the inhibitor. Subsequent conformational changes stabilize the covalent inhibitory complex. Some of the initial acyl-enzyme intermediates fail to complete the stabilizing conformational change and undergo deacylation resulting in the release of the free enzyme and the cleaved inhibitor. Because of the covalent linkage, the protease-inhibitor complex is SDS-resistant and can be readily identified on SDS-polyacrylamide gels. We have tested the interaction of a commercial α1-antitrypsin preparation (Sigma) with ELA2A and ELA2B. SDS-PAGE analysis of the reaction between ELA2A and α1-antitrypsin revealed both the covalent complex and the cleaved, dissociated inhibitor. In contrast, neither complex formation nor cleavage of α1-antitrypsin was detectable with ELA2B (Fig 2B).

Chimeras between ELA2A and ELA2B. In order to identify the evolutionary amino-acid changes that inactivated ELA2B, first we have engineered chimeras between ELA2A and ELA2B. Arbitrarily, we have divided the ELA2 sequence into three segments (amino-acids 17-100, 101-210 and 211-269) and made 4 different combinations between these segments (Fig 3). Interestingly, 3 of the 4 chimeras were completely inactive, and the only chimera with partial activity was ELA2A with the 101-210 sequence replaced with ELA2B. These observations indicate that multiple evolutionary mutations that spread across the entire primary structure inactivated ELA2B. The most relevant mutations appear to be localized in the N-terminal and C-terminal regions.

Effect of ELA2B-specific point mutations on ELA2A. To test the notion that multiple unrelated substitutions are responsible for the inactivity of ELA2B, we have converted select amino-acids in ELA2A to their ELA2B-like counterpart. Introduction of mutations V29M-V30L or S210C into ELA2A abolished elastase activity (Fig 4). In contrast, elastase activity was unaffected by mutations R79G or R87Q, while N114D caused about 50 % reduction. These observations demonstrate that not all amino-acid changes in ELA2B are deleterious, and clearly identify Met29-Leu30 and Cys210 as inactivating evolutionary mutations in ELA2B.

DISCUSSION

Here we report that the recombinant putative human elastase ELA2B has no elastase activity or measurable proteolytic activity. The finding is surprising, since native or recombinant ELA2A, which shares 90 % sequence identity with ELA2B, exhibits both elastinolytic and proteolytic activity on a variety of test substrates. Chimeras and point-mutations made between the two ELA2 isoforms indicated that multiple evolutionary mutations inactivated ELA2B. When the V29M-V30L double-mutation or the S210C mutation were introduced into ELA2A, essentially complete loss of activity was observed, confirming that Met29-Leu30 (Met16-Leu17 in chymotrypsin numbering, chy#) and Cys210 (chy# Cys189) are some of the inactivating mutations present in ELA2B.

The positions of Met29-Leu30 are occupied by Val-Val in ELA2A and the majority of the vertebrate elastases and chymotrypsins. Mutagenesis studies on rat trypsinogen, which contains Ile-Val at these positions, indicated that hydrophobic interactions by these two residues play an essential role in the transition from zymogen to active enzyme [14]. Trypsinogen is converted to trypsin by the removal of the activation peptide from the N terminus, which permits formation of a salt bridge between the newly formed N-terminal Ile

residue and Asp194 (chy#). Formation of this salt bridge induces a conformational change in the so-called activation domain of trypsin, creating the S1 specificity pocket and oxyanion hole. It is conceivable that the somewhat larger side-chains of the Met-Leu N terminus in ELA2B hinder the formation of the salt-bridge with Asp194 and/or the subsequent conformational changes of the activation domain.

Cys210 should be located at the bottom of the S1 specificity pocket if ELA2B were a true elastase. This residue is Ser189 (chy#) in elastases and chymotrypsins, while trypsins contain Asp189 (chy#). ELA2B contains 10 conserved Cys residues which form 5 disulfide bridges leaving Cys210 unpaired. The presence of an unpaired Cys may lead to misfolding through mismatching of the Cys pairs. In this regard, the proximity of Cys212 (chy# Cys191) invites speculation that Cys210 and Cys212 might compete for pairing with Cys243 (chy# Cys220). While this study clearly identified Met29-Leu30 and Cys210 as some of the inactivating mutations in ELA2B, additional mutations must also contribute to the inactivity of ELA2B, because restoration of Val29-Val30 and/or Ser210 failed to re-activate ELA2B (not shown).

From these observations it is clear that ELA2B is not an elastase enzyme. In all likelihood, *ELA2B* is a pseudogene, which can still express mRNA and possibly protein, too. On the other hand, it is also conceivable that ELA2B has a yet unidentified, specialized protease activity or its function is completely unrelated to proteolysis. The lack of proteolytic activity explains why the ELA2B protein has remained undetected in the human pancreas. Curiously, a recent large-scale proteomics approach cataloging proteins of a human pancreatic tissue extract did not find ELA2B either, however, in this study ELA2A was also missed [15]. There seems to be abundant ELA2B mRNA expressed and the 5′ regions are essentially identical between ELA2A and ELA2B, suggesting that efficient translation of ELA2B is likely to occur. In our laboratory, experiments are currently underway to develop ELA2B-specific antibodies to study pancreatic expression and localization of ELA2B.

The inactivity of ELA2B is another example of evolutionary elastase silencing in humans. It is not readily apparent why elastase expression is curtailed in the human pancreas. The primary function of elastase is to digest elastin and other dietary proteins. It is conceivable that the demand for elastin breakdown is reduced in humans relative to other vertebrates, and expression of ELA2A alone is sufficient. A comparative analysis of elastase expression and activity in vertebrate pancreata is required to address this question.

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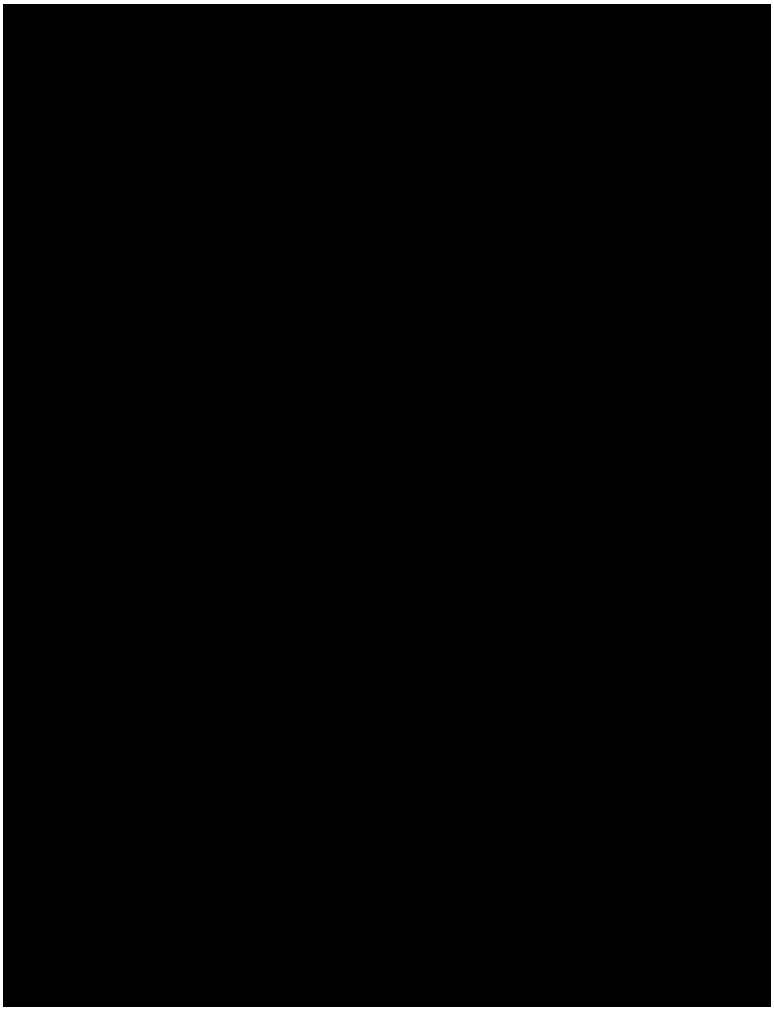

Figure 1.

Elastase activity of recombinant ELA2A (circles) and ELA2B (triangles). Recombinant human anionic trypsin (PRSS2) or cationic trypsin (PRSS1) were used to activate proelastase preparations. **A**. Proelastase 2A and 2B (600 nM) were activated with 50 nM trypsin (final concentrations) in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 2 mg/mL bovine serum albumin, in 100 μL final volume at 37 °C. After 10 min incubation, 5 μL aliquots were removed and mixed with 95 μL DQ-elastin substrate in the same buffer, and time-courses of the relative fluorescence change were recorded at 22 °C. Final elastase and substrate concentrations in the assay were 30 nM and 50 μg/mL, respectively. **B**. Proelastase 2A and 2B (300 nM) were activated with 80 nM trypsin (final concentrations) at 37 °C in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 2 mg/mL bovine serum albumin, in 800 μL final volume. At indicated times 50 μL aliquots were removed and mixed with 150 μL Glt-Ala-Ala-Pro-Leu-p-nitroanilide substrate in the same buffer and the initial rate of p-nitroaniline liberation was measured at 22 °C. Final elastase and substrate concentrations in the assay mix were 75 nM and 0.375 mM, respectively. Elastase activity was expressed as percent of maximal activity, which corresponded to a rate of 85 nM s^{-1} p-nitroaniline release.

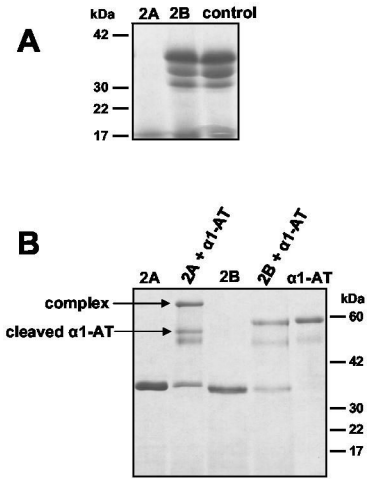

Figure 2.

Digestion of bovine milk proteins (**A**) and complex formation with α1-antitrypsin (**B**) by recombinant ELA2A and ELA2B. **A**. Proelastase 2A and 2B (200 nM) were activated with 40 nM human anionic trypsin (final concentrations) in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 2 mg/mL bovine serum albumin, for 30 min at 37 °C. Trypsin was then inactivated by adding human pancreatic secretory trypsin inhibitor to 200 nM final concentration. In a control "mock" activation reaction proelastase was omitted and only trypsin and trypsin inhibitor was included. Aliquots (25 μL) of the activation reactions were mixed with 225 μL milk-protein solution (5 mg/mL nonfat extra grade commercial dry milk dissolved in water) and incubated at 37 °C overnight. Aliquots (20 μL) were analyzed by 13 % SDS-PAGE under reducing conditions. **B**. Complex formation with α1-antitrypsin (α1-AT). Proelastase 2A and 2B were activated with 10 nM human anionic trypsin (final concentration) in 20 mM Tris-HCl (pH 8.0), 50 mM NaCl and 1 mM EDTA for 40 min at 37 °C, and 30 μg α1-antitrypsin (Sigma) was added to the activation reactions and incubated for 30 min at 22 °C. The molar ratio for ELA2: α1-AT were 1: 2.3. Samples were precipitated with 10 % trichloroacetic acid, and analyzed by 13 % SDS-PAGE under reducing conditions. The protein markers used in these experiments were MultiMark pre-stained standards from Invitrogen.

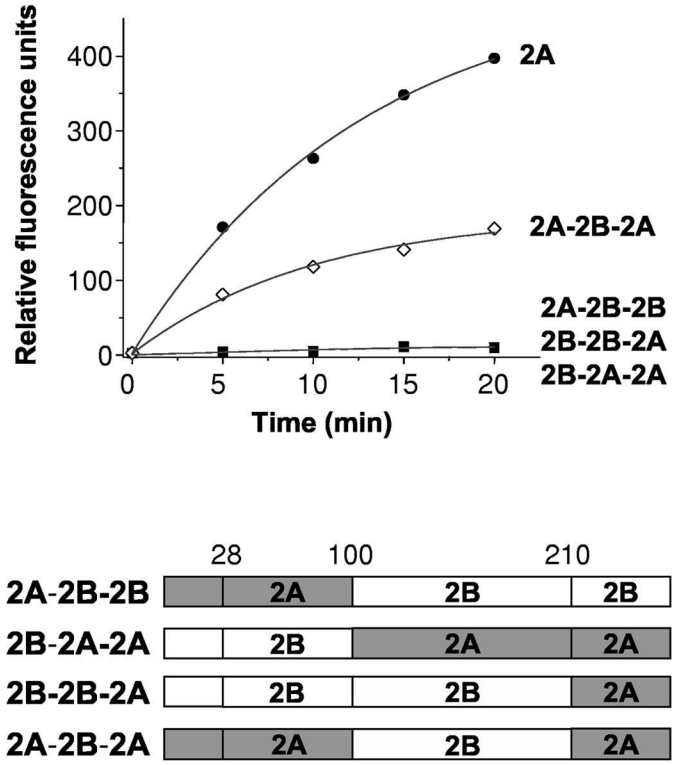

Figure 3.

Elastase activity of ELA2A-ELA2B chimeras. The fusion junctions between the indicated ELA2A (grey) and ELA2B (white) regions were at amino-acids 100 and 210. Chimeric proelastases (350 nM) were activated with 40 nM anionic trypsin (final concentrations) in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 2 mg/ml bovine serum albumin for 30 min at 37 °C. Aliquots (10 μL) were mixed with 90 μL DQ-elastin substrate in the same buffer, and time-courses of the relative fluorescence change were recorded at 22 °C. Final elastase and substrate concentrations in the assay were 35 nM and 50 μg/mL, respectively.

Figure 4.

Elastase activity of ELA2A mutants. Proelastase 2A mutants (300 nM final concentration) were activated with 20 nM anionic trypsin in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 2 mg/ml bovine serum albumin for 30 minutes at 37 C°. Aliquots (10 μL) were mixed with 90 μL DQ-elastin substrate in the same buffer, and time-courses of the relative fluorescence change were recorded at 22 °C. Final elastase and substrate concentrations in the assay were 30 nM and 50 μg/mL, respectively.