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A Solubilized Elastin Substrate for Continuous Fluorimetric Assay of Kinetics of Elastases

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Abstract

Elastolysis is central to progression of emphysema and aortic aneurysms. Characterization of steady-state enzyme kinetics of elastolysis is fettered by the insolubility of mature elastin and polydispersity of solubilized elastin. We prepared a fluor-tagged, 100 kDa fraction (fEln-100) from commercial α -elastin. It is soluble, less heterogenous in mass, cross-linked like mature elastin, and likely to retain the capacity of α -elastin to self assemble. fEln-100 has introduced the ability to compare quantitatively the *apparent* k_{cat} and K_m of elastases. For example, metalloelastase (MMP-12) displays higher apparent affinity for fEln-100, while MMP-2 displays faster catalytic turnover.

Keywords

elastin degradation; fluorescence; enzyme kinetics; elastase; protease

Elastin fibrils provide the elastic recoil of arteries and lungs, where they contribute around half or a quarter of the protein mass, respectively [1]. Degradation of elastin is integral to chronic obstructive pulmonary disease (COPD) and abdominal aortic aneurysm, conditions with high mortality among smokers [2]. Elastin degradation products in COPD potentially recruit monocytes to the lung where they differentiate into alveolar macrophages [3]. Elastin fragments promote chronic inflammation [2], encourage emphysema, and can be generated by proteases such as neutrophil elastase and macrophage metalloelastase (MMP-12) [4]. Other elastolytic proteases active in COPD include serine proteases cathepsin G and proteinase 3, MMP-9, and cysteine proteases such as cathepsins S, L, and K [5]. In aortic aneurysms, MMP-12 and -14 actively degrade the elastin of the aorta [6;7]. MMP-2 and -9 are also crucial [8] and are elastases [9]. Gaining the ability to characterize the kinetics of elastin degradation by such proteases will provide new opportunities in enzymology and pathophysiology.

Mature elastin fibrils are insoluble and intertwined with collagen, fibulin, and carbohydrates [10;11]. Elastin comprises repetitive sequences and multiple chains cross-linked by desmosine linkages formed among lysine residues modified by lysyl oxidase [10;11]. Peptide release from insoluble elastin particles can be monitored optically [12], but cannot provide steady-state enzyme kinetics in solution, is cumbersome in discontinuous sampling of time points, and is subject to variability. Commercial preparations of solubilized elastin are very heterogeneous in mass, migrating in SDS-PAGE as a continuum of bands ranging from highest to lowest apparent masses recognizable. This renders them unfit for measuring k_{cat} and K_m .

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α -elastin [10] is a soluble alternative substrate for discontinuous assays that are more sensitive and reproducible [13]. α -elastin possesses physiological cross-linking [10] and the ability to form at 37 °C filaments and fibrils resembling elastin [14]. α -elastin may be less assembled and less rigid at room temperature. α -elastin may average 17 cross-linked chains and 60 to 84 kDa [10]. We sought a tool to measure *apparent* k_{cat} and K_m of elastolysis for investigating how an MMP digests elastin. We report preparation, characterization, and application of a fluor-tagged α -elastin substrate with properties and convenience suitable for quantitative comparison of steady-state kinetics of elastin turnover in structure-function studies of MMPs.

We chose to modify α -elastin for self-quenched FRET detection. α -elastin (Elastin Products Co) is extracted from bovine neck elastin with oxalic acid until dissolved [10]. α -elastin was conjugated with BODIPY FL (Invitrogen), which has advantages over fluorescein of greater Förster radius of 57 Å [15] for better self-quenching, greater photostability [16], and greater intensity. Its succinimidyl ester was used to condense it with free lysines and α -amino termini among cross-linked chains of α -elastin. BODIPY FL was dissolved in anhydrous DMSO, added drop-wise to a solution of α -elastin (10 mg/ml), and stirred in darkness at 25 °C for 1h. The mixture was separated in darkness by size exclusion using a HiLoad 16/60 Superdex 200 column (GE Healthcare) operated with a BioRad BioLogic Duo Flow chromatography system. The fraction migrating on SDS-PAGE with apparent mobility around 125 kDa was selected as the substrate due to its full size, abundance, and susceptibility to catalytic turnover. The labeling with BODIPY was heavy enough for the fluors to be close enough to quench one another. This is convenient for monitoring proteolysis that separates fluors, resulting in increase of fluorescence (Fig. 1). The excitation maximum lies at 506 to 507 nm. The emission maximum lies near 515 nm (Fig. 1a). The proximity of these maxima is optimal for self-quenching but requires resolution by the instrument. (A donor-acceptor pair with better separated maxima will require conjugating the quencher with chemically distinct groups.) Recommended wavelengths for progress curves are ~496 nm for excitation and ~518 nm for emission using a monochromator-based fluorimeter or plate reader, or 490 and 520 nm, respectively, using a filter-based reader.

Radioimmunoassay [17] measured 46.5 nmoles of desmosine per mg of this fraction when prepared without fluor. This corresponds to 2.4% (w/w) desmosine, which is similar to reports of 1.9% (w/w) desmosine for solubilized bovine neck elastin [18] and 1.5% for insoluble bovine elastin [1]. This heavy cross-linking distinguishes mature elastin from tropoelastin. (Tropoelastin is the immature, soluble form that once cross-linked forms elastin.) 2D gel electrophoresis of this fraction prepared without fluor suggests most of the species to be 100 ± 25 kDa and have pI of 4.7 to 5.7 (Supplementary Fig. S1). This suggests that most lysines may have been modified into cross-links. More dilute species ranging in pI from 4 to 7 indicates further variability in charge, probably resulting from variability in numbers of free lysine residues. This may result from progressive incorporation of lysines into cross-links during maturation of elastin [19], or from varying numbers and sequences of chains being cross-linked. Heterogeneity is intrinsic to tropoelastin [20] and α -elastin [21]. The fractionation by gel filtration narrowed the MW distribution substantially enough (Supplemental Figures) to be used in approximating *apparent* k_{cat} and K_m values within $\pm 25\%$. MALDI TOF mass spectrometry indicated the species conjugated with BODIPY to be distributed around a maximum at 95 kDa (Supplemental Fig. S2). The substrate is designated fEln-100, referring to its fluorescence and size range.

The fluorescence changes of fEln-100 upon proteolysis support analysis of *apparent* steady-state enzyme kinetics, as illustrated for MMP-12 and -2 (Fig. 1, 2). We fit progress curves by a procedure that economizes on number of reactions and that avoids complications from non-linear concentration dependence of fluorescence [22]. k_{cat}/K_m is fitted to a progress curve collected under pseudo first-order conditions ($[S] \ll K_m$) and subsequently serves to constrain

and guide fitting of k_{cat} and K_m . Measurement of the fluorescence plateau reached upon depletion of the substrate is also needed for the fits. Since proteolytic turnover of fEln-100 is slow, the reaction vessel or plate is covered to prevent evaporation.

A single reaction phase does not adequately fit entire progress curves from MMP-12 (metalloelastase) digestion of fEln-100 (Fig. 2A). Addition of a second kinetic phase provides excellent fits and residuals (Fig. 2B). The early and late kinetic phases could be fitted with separate apparent k_{cat}/K_m values via four steps of fitting (using Origin 8.1) that are illustrated in Supplementary Methods. This involved measurement of the plateau after 1 to 2 days and fitting of the plateau and apparent k_{cat}/K_m of the early phase. Initial fits were followed by refined fits. We interpret the kinetic phases in light of MMP-12 hydrolyzing ≥ 36 sites in elastin [23] and generating proteolytic products from fEln-100 with a broad range of masses (Supplemental Fig. S3). The concentration of sites hydrolyzed exceeds the protein substrate concentration $[S]$ used in calculations. The early kinetic phase probably represents hydrolysis of a population of initially accessible sites. The later phase could represent cryptic scissile bonds becoming exposed by the earlier proteolysis. The fitted k_{cat} and K_m can be regarded as *apparent* values reporting on the collection of concurrent reactions at different sites of proteolysis within fEln-100.

The early phase in progress curves with human MMP-12 catalytic domain has apparent k_{cat} of 0.013 s^{-1} , K_m of $1.3 \text{ }\mu\text{M}$, and k_{cat}/K_m of $10,000/\text{M/s}$ at 25°C (Fig. 2C). The early phase with human full-length MMP-2 has apparent k_{cat} of 0.10 s^{-1} , K_m of $4.8 \text{ }\mu\text{M}$, and k_{cat}/K_m of $21,000/\text{M/s}$ (Fig. 2D, 25°C), which approach 8-fold, 4-fold, and 2-fold higher, respectively. After addition of MMP-12 catalytic domain, lag periods of 15 to 20 min ensue before fluorescence increase from fEln-100 begins. After addition of full-length MMP-2 to fEln-100, however, fluorescence increase begins immediately but reaches lower amplitude. MMP-12 seems to require more time initially to make the scissions that separate fluor-labeled peptides from the cross-linked fEln-100 chains. These catalytic differences supplement the differences observed in regions of interaction between elastin and MMP-2 or MMP-12 [9,24].

In summary, fEln-100 possesses the heavy cross-linking of mature elastin, much less heterogeneity than the α -elastin starting material, and α -elastin's solubility and ability to self-assemble upon warming [14]. Fluorimetric detection of fEln-100 enables comparison of steady-state kinetics of its turnover quantitatively, reproducibly, and with insights from apparent k_{cat} and K_m . Its turnover by full-length MMP-2 is faster than by MMP-12 that has greater affinity for it and that may cleave more sites. fEln-100 allows good throughput for structure-function studies and screening for therapeutic leads.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

COPD	chronic obstructive pulmonary disease
fEln	fluorescent, soluble α -elastin
FRET	Förster resonance energy transfer

MALDI TOF	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MMP	matrix metalloproteinase
TNC	buffer containing Tris·HCl, NaCl, CaCl ₂ , and Brij-35

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Fig. 1.

Changes in fluorescence spectra of fEln-100 upon proteolysis by an elastase. The fluorescence emission increases upon proteolysis. 100 nM fEln-100 was exposed to 10 nM of human MMP-12 catalytic domain for 80 minutes at 25 °C in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM CaCl₂, 0.1 mM ZnCl₂, and 0.035% Brij-35 (TNC buffer). The inset shows the fluorescence excitation spectra of the same samples. The spectra were collected using a Biotek Synergy MX plate reader, with monochromator set to a 9 nm bandpass.

Fig. 2.

Fits of fluorescence progress curves of fEln-100 digested with an MMP. 400 nM of fEln-100 was incubated with the human MMP in TNC buffer at 25 °C, covered, and read from the bottom using excitation and emission wavelengths chosen from spectra such as Fig. 1. The parameters k_{cat}/K_m (A, B) or k_{cat} and K_m (C, D) were fitted to the progress curves using the monophasic [22] or biphasic procedures described in the text. Monophasic (A) and biphasic (B) fits of k_{cat}/K_m are plotted. Fitted k_{cat} and K_m are shown for just the early phase of fits to digestion by MMP-12 (C) or MMP-2 (D).