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Development of a fluorescence resonance energy transfer assay for monitoring bacterial collagenase triple-helical peptidase activity

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Abstract

Due to their efficiency in the hydrolysis of the collagen triple-helix, *Clostridial histolyticum* collagenases are utilized for isolation of cells from various tissues, including isolation of the human pancreatic islets. However, the instability of *Clostridial* collagenase I (Col G) results in a degraded Col G that has weak collagenolytic activity and an adverse effect on islet isolation and viability. A Förster resonance energy transfer (FRET) triple-helical peptide (fTHP) substrate has been developed for selective evaluation of bacterial collagenase activity. The fTHP [sequence Gly-mep-Flp-(Gly-Pro-Hyp)₄-Gly-Lys(Mca)-Thr-Gly-Pro-Leu-Gly-Pro-Pro-Gly-Lys(Dnp)-Ser-(Gly-Pro-Hyp)₄-NH₂] had a melting temperature (T_m) of 36.2 °C and was hydrolyzed efficiently by bacterial collagenase ($k_{cat}/K_M = 25,000 \text{ sec}^{-1}\text{M}^{-1}$), but not by clostripain, trypsin, neutral protease, thermolysin, or elastase. The fTHP bacterial collagenase assay allows for rapid and specific assessment of enzyme activity towards triple-helices and thus potential application for evaluating the efficiency of cell isolation by collagenases.

Keywords

bacterial collagenase; clostripain; collagen; FRET protease assay; islet isolation; stem cell isolation

Introduction

Clostridium histolyticum is an anaerobic bacteria that produce two classes of collagenases [1, 2]. *Clostridial* collagenases are responsible for bacterial invasion of the host in gas gangrene. *Clostridial* collagenases are amongst the most potent collagenolytic enzymes, and in comparison to the mammalian collagenases [i.e., matrix metalloproteinases (MMPs)], have multiple cleavage sites within the collagen triple-helix. Class I *Clostridial* collagenase

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(Col G) cleaves interstitial (types I–III) collagen initially near the *N*-termini, whereas class II (Col H) cleaves interstitial collagen near the middle to produce 35 and 62 kDa fragments [3, 4]. Col G is the more efficient enzyme for hydrolysis of intact collagen triple-helices [5, 6].

Clostridial collagenases are used in a wide variety of biotechnological applications. Due to their efficiency in the hydrolysis of the collagen triple-helix, *Clostridial* collagenases are utilized for isolation of cells from various tissues, including adipocytes, hepatocytes, and other cells from lung, epithelium and adrenal tissue; cardiomyocytes and other cells from bone, cartilage, muscle, thyroid, and endothelium; and human and porcine pancreatic islets [7]. Of great interest and practical value is the isolation of the human pancreatic islets, which are used in transplantation for type I diabetes treatment. However, the isolation process often results in low islet yield. A notable factor responsible for this problem is the inconsistent collagenolytic activity between different batches of collagenase [8]. This is due to the unstable nature of collagenase I/Col G, as the intact enzyme is autocatalytically degraded to the less active Ib form [9]. Degraded Col G has weak collagenolytic activity, and has an adverse effect on islet isolation and viability [9, 10].

There are several assays that assess the activity (and hence quality) of the *Clostridium histolyticum* collagenases. These assays utilize collagen (gels or fluorescein-labeled fibrils), denatured collagen [dye-bound gelatin (azocoll)], or synthetic substrates based on collagen sequences [*N*-[3-(2-furyl)acryloyl]-Leu~Gly-Pro-Ala (FALGPA), (4-phenylazocarbobenzoxy)-Pro-Leu-Gly-Pro-Arg (PZ peptide), or Dabcyl- γ Abu-Lys-Gly-Gly-Pro-Leu~Gly-Pro-Pro-Gly-Pro-Gly-Gly-Cys(Aedans)-Lys-NH₂ (where Dabcyl is 4-((4-(dimethylamino)phenyl)azo)-benzoyl and Aedans is 5-[2-(acetamido)ethylamino]naphthalene-1-sulfonic acid)] [5, 11–13]. Due to the instability of Col G resulting in decreased collagenolytic activity, it is desirable to utilize an assay that monitors the triple-helical peptidase activity of the enzyme in order to evaluate its integrity. Azocoll and synthetic substrates monitor activities towards linear substrates, while collagen gel and fibril assays evaluate the collagenolytic activity of Col G or Col H. Unfortunately, collagen gel assays are non-linear, time consuming, and have poor precision, and fluorescein-labeled collagen can be a mixture of intact and denatured triple-helices (i.e., both collagen and gelatin). Ultimately, it would be desirable to develop a convenient, sensitive assay that monitors proteolytic activity towards collagen triple-helices.

We have previously described a number of Förster resonance energy transfer (FRET) triple-helical peptide (THP) substrates (fTHPs) that were either suitable for most collagenolytic MMPs or selective for different collagenolytic MMPs [14–25]. In most cases, FRET was achieved by incorporating (7-methoxycoumarin-4-yl)acetyl (Mca) as the fluorophore and 2,4-dinitrophenyl (Dnp) as the quencher. Sequences incorporated within these prior fTHPs were optimized for MMPs, and thus are not appropriate for bacterial collagenases. We have presently developed a FRET triple-helical substrate for selective evaluation of bacterial collagenase activity.

MATERIALS AND METHODS

The reagents and solvents used for peptide synthesis were obtained as follows: *N,N*-diisopropylethylamine (DIPEA), *N*-methylmorpholine (NMM), acetonitrile (HPLC grade), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), piperidine, and water (HPLC grade) from Sigma-Aldrich; *N*-hydroxybenzotriazole•H₂O (HOBt•H₂O), *N*^α-(9-fluorenylmethoxycarbonyl)-*N*^ε-(7-methoxycoumarin-4-yl)-acetyl-L-lysine [Fmoc-Lys(Mca)], and *N*^α-(9-fluorenylmethoxycarbonyl)-*N*^ε-(2,4-dinitrophenyl)-L-lysine [Fmoc-Lys(Dnp)] from Anaspec (Fremont, CA); methyl *tert*-butyl ether (MTBE) from J.T. Baker; Fmoc-amino acids, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU), and *N,N*-dimethylformamide (DMF) (AR grade) from AGTC Bioproducts (Wilmington, MA); 2,5-dihydroxybenzoic acid (DHB) and trifluoroacetic acid (TFA) from Thermo Fisher Scientific (Waltham, MA); *N*^α-(9-fluorenylmethoxycarbonyl)-4*R*-fluoro-L-proline (Fmoc-Flp) from Bachem (Torrance, CA); decanoic acid (designated C₁₀) and dithiothreitol (DTT) from Acros Organics (Geel, Belgium); and NovaPEG Rink amide resin from EMD Millipore (San Diego, CA). *N*^α-(9-fluorenylmethoxycarbonyl)-4*R*-methyl-L-proline (Fmoc-mep) and fTHP-15 [(Gly-Pro-Hyp)₅-Gly-Pro-Lys(Mca)-Gly-Gln-Pro-Gly-Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)₅-NH₂] were synthesized as described [19, 22, 26].

The enzymes and reagents for enzyme assays were acquired as follows: collagenase (CLSPA, a mixture of Col G and Col H), clostripain, trypsin (TPCK-treated), neutral protease (dispace), and elastase from Worthington Biochemical Corporation (Lakewood, NJ); thermolysin and *N*^α-tosyl-Lys-chloromethyl ketone hydrochloride (TLCK) from Sigma-Aldrich (St. Louis, MO); and Pierce bicinchoninic acid (BCA) Protein Assay Kit from Thermo Scientific (Rockford, IL).

Peptide synthesis

Peptides were synthesized with Fmoc chemistry using the PS3 Peptide synthesizer (Protein Technologies, Tuscon, AZ) for substrate **1** and the Liberty Microwave Peptide Synthesizer (CEM Corporation, Mathews, NC) for the remainder of the substrates. For substrate **1** all amino acid couplings were performed with 4 eq excess of Fmoc-amino acid, 3.9 eq of HCTU/HOBt, and 8 eq of NMM and carried out for 1 h. NovaPEG Rink amide resin (0.67 mmol/g; 150 mg) was utilized. Microwave assisted couplings of Fmoc-amino acids for substrates **2**, **3**, and **4** were performed with 5 eq of each amino acid, 4.9 eq of HCTU/HOBt, and 10 eq of NMM with the exception of Fmoc-Flp and Fmoc-mep, where 3 eq excess of Fmoc-amino acid, 2.9 eq HCTU/HOBt, and 6 eq of NMM were used. Coupling reactions were performed for 5 min at 50 °C and 25 W with the exception of Fmoc-Lys(Mca) and Fmoc-Lys(Dnp), where the coupling reaction was extended to 20 min, and Fmoc-Flp and Fmoc-mep, where the coupling reaction was carried out for 45 min. NovaPEG Rink amide resin (0.48 mmol/g; 210 mg) was utilized.

Removal of the Fmoc group during the synthesis of substrate **1** was performed 3 times for 6 min each with 5% piperidine, 1% DBU in DMF. Microwave assisted removal of the Fmoc group using 10% piperazine in DMF was performed in two cycles. The initial deprotection

was carried out for 30 sec (75 °C, 35 W), followed by deprotection for 180 sec (75 °C, 35 W).

Decanoic acid was coupled to the *N*-terminal Gly using 4 eq of decanoic acid, 3.9 eq HCTU, and 8 eq DIPEA for 1 h. Peptides were cleaved from the resin with TFA–H₂O–thioanisole (90:5:5) in an Ar atmosphere for 3 h, precipitated from cold MTBE, and centrifuged. The resulting pellets were dissolved in water and lyophilized.

Peptides were purified using RP-HPLC (Agilent 1260 Infinity with multi wavelength detector) on a Vydac C₁₈ column (15–20 µm, 300 Å, 250 × 22 mm) at a flow rate of 10 mL/min and detection set at λ = 220 and 324 nm. Substrate **1** was purified using a gradient of 5–80% B over 80 min (where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile), substrates **2** and **3** in 20–50% B over 60 min, and substrate **4** in 25–55% B in 60 min. Collected fractions were analyzed by MALDI-TOF mass spectrometry (Voyager-DE™ PRO) and RP-HPLC (Agilent 1260 Infinity) with detection at λ = 220 and 324 nm, on a Vydac C₁₈ column (5 µm, 300 Å, 150 × 4.6 mm). The analytical gradient was 2–98% B over 20 min, flow rate = 1 mL/min. HPLC homogenous fractions were combined and freeze-dried. MS analyses of substrates **1**, **2**, **3**, and **4** resulted in [M+H]⁺ = 3649.5 Da (theoretical 3645 Da), 3619.5 Da (theoretical 3619 Da), 3613.0 Da (theoretical 3614 Da), and 3768.1 Da (theoretical 3769 Da), respectively.

Circular dichroism (CD) spectroscopy

CD spectra were obtained over the range of λ = 180–250 nm using a 0.1 cm quartz cuvette on a JASCO J-810 spectropolarimeter. All peptides were dissolved in 1% aqueous acetic acid at a concentration of 20–25 µM and left overnight at 4 °C to allow the formation of the triple-helix. The thermal transition curve was obtained by recording the molar ellipticity ([θ]) at λ = 225 nm with temperature increasing by 20 °C/h from 5–80 °C. Temperature was controlled by a JASCO PTC-348WI temperature control unit. The melting temperature (*T*_m) was defined as the inflection point in the transition region. The spectra were normalized by designating the highest [θ]_{225nm} as 100% folded, and the lowest [θ]_{225nm} as 0% folded [27–30].

BCA protein assay

The concentration of all enzymes was determined with the Pierce BCA Protein Assay using bovine serum albumin (BSA) as standard and following the manufacturer's instructions. BSA standards were prepared in a concentration range of 0–2000 µg/mL, and working reagent was prepared by mixing reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1 M sodium hydroxide) with reagent B (containing 4% cupric sulfate) in a ratio of 50:1 (v/v). The working reagent was mixed with BSA standards or sample (in a 96-well plate) in a ratio of 8:1 (v/v), incubated at 37 °C for 30 min, and absorbance read at λ = 562 nm on a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek). All measurements were done in triplicate. The average absorbance of the standards was plotted over BSA concentration to generate a standard curve for determination of sample protein concentration.

FRET based enzyme activity assays

Immediately after the BCA assay, each enzyme was diluted with Tris salt buffer (TSB) (50 mM Tris, 100 mM NaCl, 10 mM CaCl₂, 0.05% Brij-35, 0.02% NaN₃, pH 7.5) to a final concentration of 0.045 μ M in the FRET assay. Clostripain was activated by adding DTT to the buffer (see below). Peptides were dissolved in TSB, diluted to desired concentrations over the range of 0.1 – 25 μ M (as measured by UV-Vis spectroscopy), and left overnight at 4 °C to allow the proper folding of the peptide triple-helix. Enzymes were incubated with substrates at 25 °C, and fluorescence was monitored continuously at $\lambda_{\text{excitation}} = 324$ and $\lambda_{\text{emission}} = 394$ nm for 10 min, and a final measurement was performed after 24 h. All measurements were done in triplicate on a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek).

To determine rates of hydrolysis (μ M/sec), fluorescence (RFU) was plotted over time (sec), using data points from only the linear portion of the hydrolysis curve. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis (24 h), and then multiplied by the substrate concentration (μ M) to obtain rates of hydrolysis in units of micromolar per second. For reactions of collagenase with each substrate, kinetic parameters were calculated using Lineweaver-Burke, Hanes-Woolf, and Eadie-Hofstee linear plots and by nonlinear regression, Michaelis-Menten model, with GraphPad Prism 5 software.

DTT/TLCK experiments

After the BCA assay, collagenase and clostripain were divided into two samples each. One sample was diluted with TSB, and DTT was added to the second one to a final concentration of 10 mM. Enzymes were incubated with DTT for 3 h at 4 °C. Both batches were further divided into two samples and TLCK (clostripain inhibitor) was added to one of them to a total concentration of 1 mM and incubated for 1.5 h at 4 °C. All four samples were assayed as described above with substrate **2** and fTHP-15.

Mass spectrometric analysis of cleavage sites

Peptides and either collagenase or clostripain were prepared as described above, with the exception that the buffer used did not contain Brij-35 and NaN₃. Spectra were obtained after 1 h of enzymatic reaction, using DHB as the matrix on a Voyager-DETM PRO instrument.

RESULTS AND DISCUSSION

We have presently pursued the design of a selective substrate to monitor the collagenolytic activity of *Clostridial* collagenases. One of the most convenient ways to assess the activity of proteases is a FRET-based assay. The Mca fluorophore/Dnp quencher pair is widely used in protease research, and both Mca and Dnp are commercially available as Fmoc-Lys-modified building blocks. Designed substrates had four Gly-Pro-Hyp tripeptide units on both sides of the putative cleavage site sequence, as Gly-Pro-Hyp repeating tripeptides stabilize the triple-helix [26].

Ultimately, four peptides were synthesized as potential substrates for bacterial collagenases. All peptides were evaluated for the thermal stability of the triple-helix using CD spectroscopy and tested with commercially available collagenase and several other proteases in a series of FRET-based activity assays. The other proteases examined were clostripain, trypsin, neutral protease (dispase), elastase, and thermolysin. These proteases were chosen to assess (i) the selectivity of the potential substrate and (ii) the usefulness of the substrate in measuring the activity of collagenase in different commercially available crude enzyme preparations [7]. The latter activity is particularly important because collagenase is mostly available as a crude protein or a mixture of enzymes for tissue dissociation (i.e., Liberase) [7]. Thus, the enzymes chosen herein to test along with collagenase are representative of those often found as part of the commercially available collagenase blends.

Substrate **1** was (Gly-Pro-Hyp)₄-Gly-Lys(Mca)-Thr-Gly-Pro-Leu-Gly-Pro-Lys-Gly-Lys(Dnp)-Ser-(Gly-Pro-Hyp)₄-NH₂. The bacterial collagenase labile sequence was based on the preference of bacterial collagenases for Leu-Gly bonds [8] and the human $\alpha 1(\text{II})246-253$ sequence (Thr-Gly-Pro-Leu-Gly-Pro-Lys-Gly). CD spectroscopic analysis of substrate **1** suggested a modest triple-helical structure (Figure 1). The CD spectrum was characteristic of a triple-helix, with a maximum at $\lambda = 225$ nm and a minimum at $\lambda = 200$ nm, but the signal intensity was very weak. Additionally, there was no distinguishable thermal transition curve (Figure 2). Substrate **1** proved to be a good substrate for collagenase, with a $k_{\text{cat}}/K_{\text{M}}$ value of $30,300 \text{ sec}^{-1}\text{M}^{-1}$ (Figure 3, Table 1). However, substrate **1** was very susceptible to cleavage by trypsin (Figure 4) due to the presence of Lys in the sequence. Activity with clostripain was also observed (Figure 4). Thus, substrate **1** did not have satisfactory selectivity and formed a triple-helix of low stability. The high proteolytic activity of collagenase towards this substrate was mostly likely due to the ability of Col H to hydrolyze denatured triple-helices [6].

Substrate **2** was (Gly-Pro-Hyp)₃-Gly-Pro-Flp-Gly-Lys(Mca)-Thr-Gly-Pro-Leu-Gly-Pro-Pro-Gly-Lys(Dnp)-Ser-Gly-Pro-Flp-(Gly-Pro-Hyp)₃-NH₂. To remove trypsin activity, the Lys from substrate **1** was replaced by Pro in substrate **2**. Pro in the P₃' subsite is favored by bacterial collagenases [6, 11]. The Gly-Pro-Hyp tripeptides closest to the N- and C-terminus of the cleavage site were replaced with Gly-Pro-Flp tripeptides to improve the stability of the triple-helix [31]. The CD spectrum of substrate **2** had greater triple-helical character compared with substrate **1** (Figure 1), in that the signal was stronger. Melting curve analysis (Figure 2) indicated a T_{m} of 27 °C. Substrate **2** was an efficient collagenase substrate, with $k_{\text{cat}}/K_{\text{M}} = 20,800 \text{ sec}^{-1}\text{M}^{-1}$ (Figure 3, Table 1). Substrate **2** was not cleaved by any of the other enzymes except for clostripain (Figure 5). Ultimately, substrate **2** was deemed to have too low a T_{m} value.

Substrate **3** was Gly-mep-Flp-(Gly-Pro-Hyp)₄-Gly-Lys(Mca)-Thr-Gly-Pro-Leu-Gly-Pro-Pro-Gly-Lys(Dnp)-Ser-(Gly-Pro-Hyp)₄-NH₂. The Lys from substrate **1** was replaced by Pro in substrate **3**, and a Gly-mep-Flp tripeptide was added to the N-terminus of the peptide to improve triple-helix stability [32]. Alternatively, a lipophilic C₁₀ chain was added to the N-terminus instead of Gly-mep-Flp, as alkyl chains can stabilize triple-helices [33]. The resulting substrate **4** [C₁₀-(Gly-Pro-Hyp)₄-Gly-Lys(Mca)-Thr-Gly-Pro-Leu-Gly-Pro-Pro-Gly-Lys(Dnp)-Ser-(Gly-Pro-Hyp)₄-NH₂] had very limited water solubility and was not

further tested. The CD spectrum of substrate **3** was characteristic of a triple-helix (Figure 1), and melting curve analysis (Figure 2) revealed $T_m = 36.2$ °C. Substrate **3** was hydrolyzed by collagenase with a $k_{cat}/K_M = 25,000 \text{ sec}^{-1}\text{M}^{-1}$ (Figure 3, Table 1). Substrate **3** was not cleaved by any of the other enzymes except for clostripain (Figure 6).

Surprisingly, enzymatic assays showed clostripain activity towards all peptides. This was anticipated for substrate **1**, which contained a Lys residue in the sequence, as clostripain is known to cleave amide bonds following Arg and Lys residues [34]. However, substrates **2** and **3** did not have Lys or Arg residues in their sequences. Particularly odd is that the same peptides were not cleaved by trypsin, despite the similar sequence specificity of trypsin and clostripain [34, 35]. To further investigate the unusual clostripain activity, substrate **2** was incubated with collagenase or clostripain and cleavage sites were evaluated using mass spectrometry (Figure 7). The most prominent fragment identified from substrate **2** following clostripain treatment (1057.4 Da) matched one of the fragments obtained from collagenase treatment (Table 2). These results suggest that the clostripain was contaminated with collagenase, which was responsible for the observed peptidase activity with substrates **2** and **3**.

To further evaluate the presence of collagenase in clostripain, fTHP-15 was incubated with either collagenase or clostripain. fTHP-15 is a substrate for collagenolytic MMPs [22], and contains potential cleavage sites for bacterial collagenase and clostripain. In the clostripain digestion of fTHP-15, mass spectrometric analysis (Figure 8, top) revealed fragments that match the sequence specificities of collagenase (hydrolysis at Hyp~Gly and Gln~Gly bonds) and clostripain (hydrolysis at Arg~Gly bonds) (Table 2). Thus, the commercially available clostripain appeared to contain both clostripain and collagenase.

The activity of collagenase and clostripain with fTHP-15 was further examined in the presence of DTT or TLCK. DTT is an activator of clostripain and an inhibitor of collagenase [34], while TLCK is an inhibitor of clostripain but has no effect on collagenase [34, 36]. The highest activity was observed for collagenase without either DTT or TLCK treatment (Figure 9). TLCK treated collagenase exhibited 88.6% activity compared with the untreated enzyme, whereas DTT treatment resulted in a significant loss of activity, 69.5% for DTT and 56% for DTT + TLCK treated enzyme compared with untreated (Figure 9). These results indicate that a small fraction of the observed activity towards fTHP-15 in the collagenase is due to clostripain contamination, as the addition of TLCK slightly decreases the activity, and it has been shown previously that TLCK does not affect *Clostridial* collagenase [36].

Activity for clostripain and DTT treated clostripain was basically identical (Figure 9). Cleavage site analysis revealed that when the clostripain sample was incubated with DTT, which should activate clostripain but inhibit collagenase [34], fTHP-15 was only cleaved at bonds corresponding to clostripain sequence preferences (i.e., Arg~Gly) (Figure 8, bottom; Table 2). The DTT treatment eliminated hydrolysis at other sites found in the untreated sample (Figure 8, top), sites which correspond to collagenase sequence preferences [8]. Thus, overall activity for clostripain and DTT treated clostripain appeared identical because

DTT addition activated the clostripain but inhibited the collagenase impurity to similar extents.

TLCK reduced the activity of clostripain (not treated with DTT) towards fTHP-15 to 82.9% and almost completely inhibited the DTT treated enzyme (5.2%) (Figure 9). Thus, fTHP-15 hydrolysis by clostripain showed that TLCK can almost completely inhibit the DTT activated enzyme. This was not observed when substrate **2** was cleaved by the clostripain preparation (data not shown), further supporting the notion that substrate **2** (as well as substrate **3**) was not cleaved by clostripain, but in fact, was cleaved by collagenase present in the commercially available clostripain. Treatment of clostripain with DTT, followed by incubation with substrate **3**, resulted in no substrate hydrolysis (Figure 6). This result additionally supports the conclusion that the “clostripain activity” observed towards substrates **2** and **3** was actually due to collagenase. The contamination of clostripain by collagenase is not surprising, as clostripain is often isolated from *Clostridium histolyticum* [34].

We have designed and synthesized a THP substrate for *Clostridium histolyticum* collagenase. Substrate **3** has good triple-helix thermal stability, is very selective considering other proteases often found in collagenase preparations, and is equipped with fluorophore and quencher allowing for measuring enzymatic activity in a FRET assay. Substrate **3** will be especially useful for laboratories that utilize collagenase for different cell isolations, including the isolation of human pancreatic β -cells (islets). Pancreatic β -cells are crucial for one of the emerging treatment strategies for type I diabetes. The present study has developed a tool by which the quality of bacterial collagenase can be assessed specifically and rapidly prior to tissue treatment, reducing the risk of inefficient β -cell isolations.

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Abbreviations

BCA	bicinchoninic acid
BSA	bovine serum albumin
CD	circular dichroism
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DHB	2,5-dihydroxybenzoic acid
DIPEA	<i>N,N</i> -diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
Dnp	2,4-dinitrophenyl
DTT	dithiothreitol
Flp	4 <i>R</i> -fluoro-L-proline

Fmoc	9-fluorenylmethoxycarbonyl
FRET	Föster resonance energy transfer
fTHP	fluorogenic triple-helical peptide
HCTU	2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate
HOBt	<i>N</i> -hydroxybenzotriazole
Hyp	4-hydroxy-L-proline
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
Mca	(7-methoxycoumarin-4-yl)-acetyl
mep	4 <i>R</i> -methyl-L-proline
MMP	matrix metalloproteinase
MTBE	methyl <i>tert</i> -butyl ether
NMM	<i>N</i> -methymorpholine
RP-HPLC	reversed-phase high performance liquid chromatography
TFA	trifluoroacetic acid
THP	triple-helical peptide
TLCK	<i>N</i> ^α -tosyl-Lys-chloromethyl ketone hydrochloride
TSB	Tris salt buffer

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Figure 1.

CD spectra of substrates **1** (blue), **2** (red), and **3** (green). Substrates were dissolved in 1% aqueous acetic acid and spectra were obtained over a range of $\lambda = 180$ –250 nm. All three spectra had characteristics of triple-helices, with a maximum at ~ 225 nm and a minimum at ~ 200 nm.

Figure 2.

(Top) Melting curves for substrate **1** (dotted dashed line), substrate **2** (solid line) and substrate **3** (dashed line). Substrates were dissolved in 1% aqueous acetic acid to concentrations of 20–25 μM and $[\theta]$ at $\lambda = 225 \text{ nm}$ was recorded with increasing temperature. (Bottom) The first derivatives (inflection points) of the melting curves indicated $T_m = 27$ and 36.2°C for substrate **2** and substrate **3**, respectively.

Figure 3.

Lineweaver-Burke analysis of collagenase hydrolysis of substrate 1, substrate 2, and substrate 3. The full analysis is shown in the top panel, while the bottom panel shows the higher substrate values for easier visualization of differences in K_M determination. Conditions are described in Materials and Methods, and results reported in Table 1.

Figure 4. Protease activity towards substrate **1**. Substrate **1** was incubated with collagenase (blue), trypsin (green), clostripain (red), neutral protease (orange), thermolysin (light blue), or elastase (yellow) and the change in fluorescence (RFU) monitored over time.

Figure 5. Protease activity towards substrate **2**. Substrate **2** was incubated with collagenase (blue), trypsin (green), clostripain (red), neutral protease (orange), thermolysin (light blue), or elastase (yellow) and the change in fluorescence (RFU) monitored over time.

Figure 6. Protease activity towards substrate **3**. Substrate **3** was incubated with collagenase (blue), trypsin (green), clostripain (red), clostripain + DTT (purple), neutral protease (orange), thermolysin (light blue), or elastase (yellow) and the change in fluorescence (RFU) monitored over time.

Figure 7. MALDI TOF mass spectrometric analysis of substrate **2** hydrolysis by collagenase (top) or clostripain (bottom).

Figure 8. MALDI TOF mass spectrometric analysis of fTHP-15 hydrolysis by clostripain without (top) and with (bottom) DTT treatment.

Figure 9. Hydrolysis of fTHP-15 by collagenase (Col) or clostripain (Clos). Both enzymes were treated with TLCK, DTT, or both TLCK and DTT, or untreated. Fluorescence (RFU) was monitored at 0 (black) and 3.5 (grey) h.

Table 1

Kinetic parameters for hydrolysis of substrates **1**, **2**, and **3** by bacterial collagenase.

Substrate	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M ($sec^{-1}M^{-1}$)
1	1.98 ± 0.15	0.060	$30,300 \pm 8,680$
2	6.34 ± 1.29	0.132	$20,800 \pm 2,420$
3	5.65 ± 2.90	0.141	$25,000 \pm 3,350$

All experiments were performed in triplicate. k_{cat} values were calculated from experimentally determined k_{cat}/K_M and K_M values.

Table 2

Mass spectrometric analysis of substrate hydrolysis by collagenase and clostripain.

Substrate	Enzyme	Fragment Sequence	MW (Da) theoretical/observed
2	collagenase	~Gly-Pro-Flp-Gly-Lys(Mca)-Thr-Gly-Pro-Leu~	1057.38/1057.20 ^a
“	“	~Gly-Lys(Mca)-Thr-Gly-Pro-Leu~	788.09/788.12 ^b
2	clostripain	~Gly-Pro-Hyp-Gly-Pro-Flp-Gly-Lys(Mca)-Thr-Gly-Pro-Leu~	1324.67/1324.56
“	“	~Gly-Pro-Flp-Gly-Lys(Mca)-Thr-Gly-Pro-Leu~	1057.38/1057.43
“	“	~Gly-Lys(Mca)-Thr-Gly-Pro-Leu~	788.09/788.30
fTHP-15	clostripain	(Gly-Pro-Hyp) ₅ -Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly-Leu-Arg~	2461.92/2462.60
“	“	(Gly-Pro-Hyp) ₅ -Gly-Pro-Lys(Mca)-Gly-Pro-Gln~	2135.52/2135.90
“	“	(Gly-Pro-Hyp) ₅ -Gly-Pro-Lys(Mca)~	1853.22/1853.80
“	“	(Gly-Pro-Hyp) ₅ ~	1354.45/1354.50 ^c
“	“	~(Gly-Pro-Hyp) ₅ -NH ₂	1352.45/1354.50 ^c
fTHP-15	clostripain + DTT	(Gly-Pro-Hyp) ₅ -Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly-Leu-Arg~	2461.92/2462.0
“	“	~(Gly-Pro-Hyp) ₅ -NH ₂	1352.45/1353.50

^a Also observed was [M + Na]⁺ = 1079.24 Da.^b Also observed was [M + Na]⁺ = 810.15 Da.^c Also observed was [M + K]⁺ = 1393.60 Da.