Enzyme-Triggered Hydrogelation via Self-Assembly of Alternating Peptides

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Experimental Section:

Materials. Boc-Lys(Boc)-OSu, leucine ethyl ester hydrochloride, trifluoroacetic acid (TFA), dichloromethane (DCM), acetonitrile (ACN), dimethyl sulfoxide (DMSO), triethylamine (TEA), hexafluoro-2-propanol (HFIP), deuteratedtrifluoroacetic acid (CF_3COOD), α-cyanohydroxycinnamic acid (CHCA, MALDI-TOF matrix) and α-chymotrypsin (from bovine pancreas, Type II, lyophilized powder, ⩾40 units/mg protein) were all purchased from Sigma-Aldrich and were used as received. Deionized water (DI, 18.2 MΩ·cm purity) was obtained from a RIOS 16/MILLQ synthesis Millipore water purification system. Crude papain (EC # 3.4.22.2; source-Carica papaya; 30 000 USP units/mg of solid; molecular weight 21K) was purchased from CalBioChem.

SyntheticMethods

General route to synthesize KL-OEt: The method used was adapted from a literature procedure.[1] Boc-Lys(Boc)-OSu (1330.5 mg, 3 mmol) and 3.3 mmol (645.8 mg) Leu-OEt·HCl were dissolved in 10 mL DMSO at room temperature. Subsequently, 0.8 mL TEA was added and the reaction mixture was stirred at room temperature for one day. Boc-KL-OEt was obtained as a precipitate by addition of the reaction solution into 20 volumes of cold water. Deprotection was

performed in 2:1 DCM/TFA (v/v) at room temperature for 18 h.The product was dissolved in 10 mL DMSO and precipitated in150 mL cold water twice to remove L-OEt.

*General procedure followed for protease-catalyzed synthesis of (KL)$_x$.*KL-OEt (4 to 12% w/w) was dissolved in 4 mL deionized water in a 20 mL glass vial. After manual pre-titration to set the monomer solution to the desired pH (between 8.5 and 9.5), protease was added, and the reaction vial was agitated by a vortex mixer such that, in 10s, the solution underwent a sol-gel transition. The hydrogel was lyophilized for two days to give a solid for further characterization.

Instrumental Methods.

Nuclear Magnetic Resonance (NMR) Spectroscopy.Proton (^1H) NMR was recorded on a Bruker *DP*X 300 spectrometer at 300 MHz. NMR experiments were performed in TFA-*d* at 10 mg/mL with a data acquisition delay of 1 s. Data were collected and analyzed by MestRe-C software. Proton chemical shifts were referenced to tetramethylsilane (TMS) at 0.00 ppm.The DP_{avg} was determined from the relative peak intensities of N-terminal α-carbonmethine protons (3.7~3.9 ppm) and internalα-carbonmethine protons (4.2~4.5 ppm).[2,3] Signals corresponding to methylene protons of lysine (–CH_2–NH_2) at 2.7–2.9 ppm and methyl protons of leucine (–CH(CH_3)-CH_3) at 0.8–1 ppm were used to confirm the co-oligomer composition (equal units of lysine and leucine).

*Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF).*MALDI-TOF spectra were obtained on an Omni-Flex MALDI-TOF mass spectrometer (Bruker Daltonics, Inc.). The instrument was operated in a positive ion reflection mode with an accelerating potential of +20 kV. The TOF mass analyzer had pulsed ion extraction. Omni-FLEX TOF control software was used for hardware control and calibration while X-mass Omni-FLEX 6.0.0 was used for data

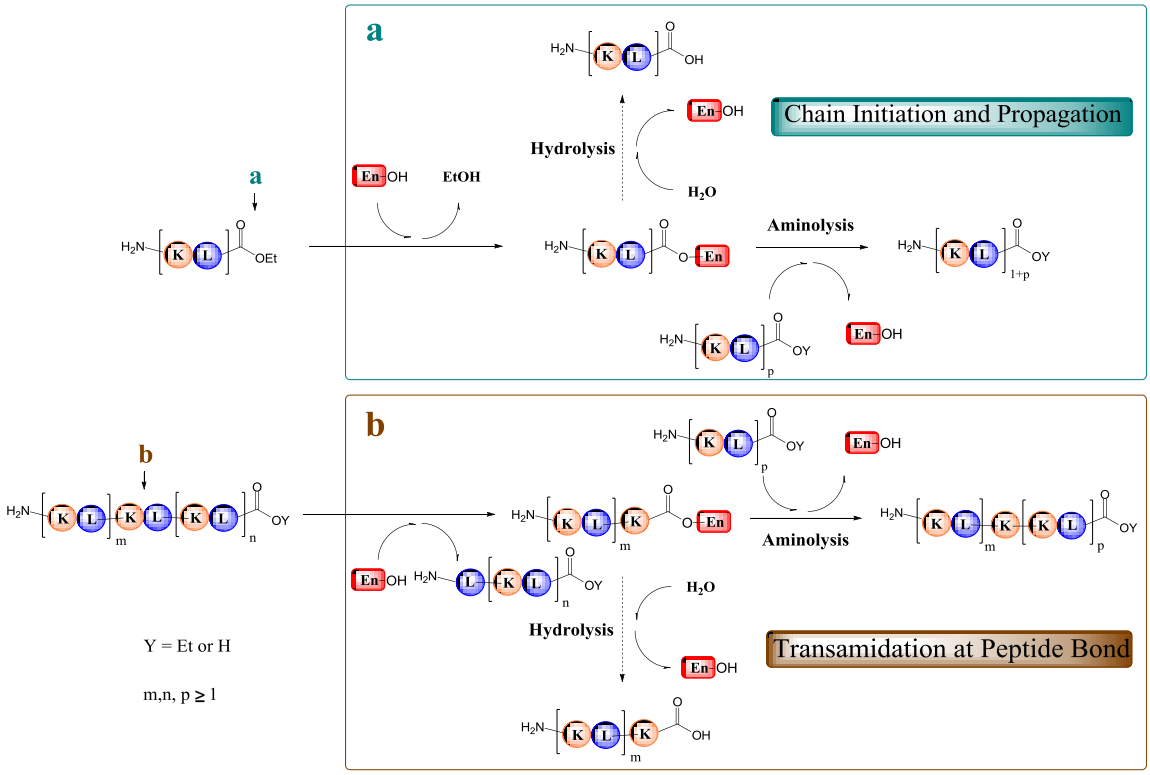
processing. Spectra were acquired by averaging at least 400 laser shots. The pulsed ion extraction delay time was 200 ns. To formulate the matrix solution, a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) was prepared in trifluoroacetic acid/acetonitrile/H_2O (TA) 0.1-to-33-to-100 v/v. Oligopeptide samples were dissolved in hexafluoroisopropanol (HFIP), diluted to 1-5 pmol/μL with TA solution and then mixed with anequal volume of a saturated matrix solution. Then, 1 μL of this mixture was applied onto the clean target which was subsequently dried using a stream of cold air. The relative intensity threshold was set so that peaks with intensity values ≤ 1% of the highest peak were considered as noise and removed from the database.

Atomic Force Microscopy (AFM). Peptides from stock solutions (1%) were diluted to a working concentration of 0.1% (w/v) from which 30μL was applied onto a freshly cleaved mica (9.9mm Diameter, TED PELLA, Inc) surface and left undisturbed for 2 min. Subsequently, the surface was washed with deionized water and dried under a gentle stream of nitrogen gas. The AFM instrument is a multimode scanning probe microscope (with Nanoscope IIIa controller and EV scanner, Veeco Instruments, Woodbury, NY). The probes used were the etched silicon probes (TESP, Bruker AFM Probes, CA) of 110-140 μm length, resonance frequency 326-347 kHz, spring constant 20-80 N/m, and tip radius 8 nm. Images were obtained in tapping mode in air at ambient temperature and ~25-65% relative humidity. Typical scanning parameters were as follows: drive frequency ~300 kHz, the root mean square voltage for the free amplitude of the probe 3 V, setpoint 1.5-2.5 V, integral and proportional gains of 0.2–0.8 and 0.4–1.6, respectively, and scan rate 1-1.5 Hz. Images were stored in 256 x 256 format and processed using the Nanoscope version 4.43r8 software. Zero-order flattening was used to measure heights. For presentation of images, first or second-order flattening was used.

Circular Dichroism (CD). CD measurements were performed at room temperature on a JASCO J-815 CD spectropolarimeter equipped with a PTC-423S single position Peltier temperature control system that was counter-cooled with an Isotemp 3016S (Fischer Scientific) water bath. Samples were loaded in a Helma 218 quartz cuvette (500 μL, 1 mm path length). Spectra from 260 nm to 190 nm were collected at 1 nm intervals with a 20 nm min^{-1} scan speed, 1 nm bandwidth and 0.25 s response. The peptide concentration was 100 μM in 10 mMTris–HCl buffer (pH 5.5-12).

Rheology. Rheological measurements were performed with a Rheometric Scientific ARESRheometer with parallel plates (diameter of 25 mm) at 25°C. Hydrogel samples were placed between parallel plates with a 1.0 mm gap. Then dynamic frequency sweep tests were carried out in the frequency range 0.1 to 15.9 Hz with a strain of 0.1% under a nitrogen atmosphere. TA Orchestrator software was used to collect and analyze data.

Results and Discussion:



Scheme S-1. Protease-catalyzed routes to oligopeptide $(KL)_x$ where En is the abbreviation for enzyme. Route (a) illustrates chain initiation and propagation from the C-terminus of peptides to form $(KL)_{1+p}$ where p ≥1; Route (b) displays intra-chain transamidation catalyzed by En-OH on substrate NH_2-$(KL)_m$-K-L-$(KL)_n$to form NH_2-L-$(KL)_n$-(C=O)-OEn and NH_2-$(KL)_m$-K-(C=O)-OY where Y=H. Subsequent aminolysis and hydrolysis reaction pathways are illustrated using NH_2-$(KL)_m$-K-(C=O)-OY as one of the substrates.

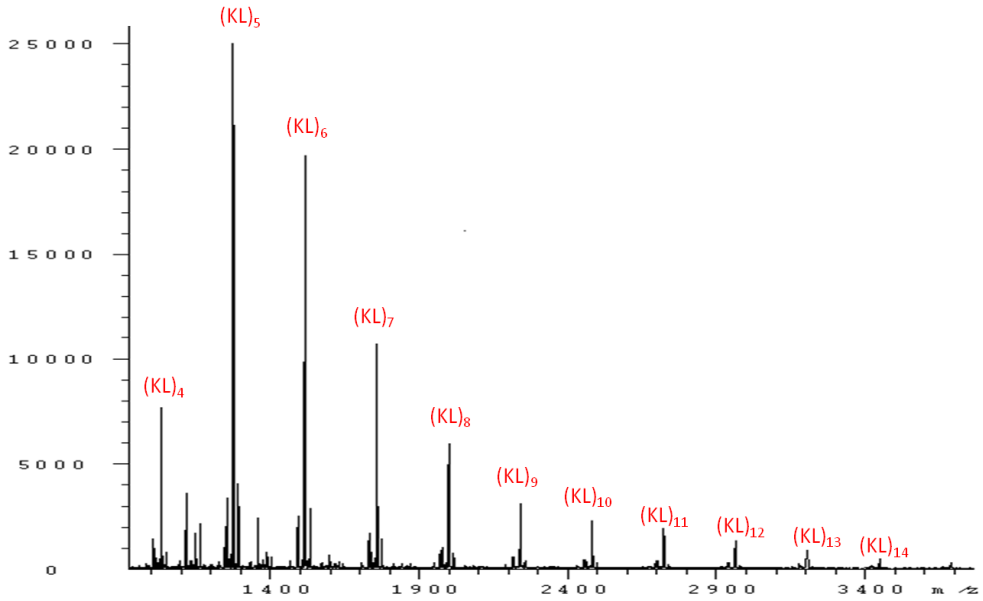


Figure S-1: MALDI-TOF spectra of alternating peptide $(KL)_x$, synthesized by α-chymotrypsin at pH 8.5 from substrate concentration 12% KL-OEt w/w. $(KL)_x$ is an abbreviation for x units of Lys-Leu dipeptide repeat units. Values of m/z observed are ± 1 Da of those expected for molecular ion peaks.

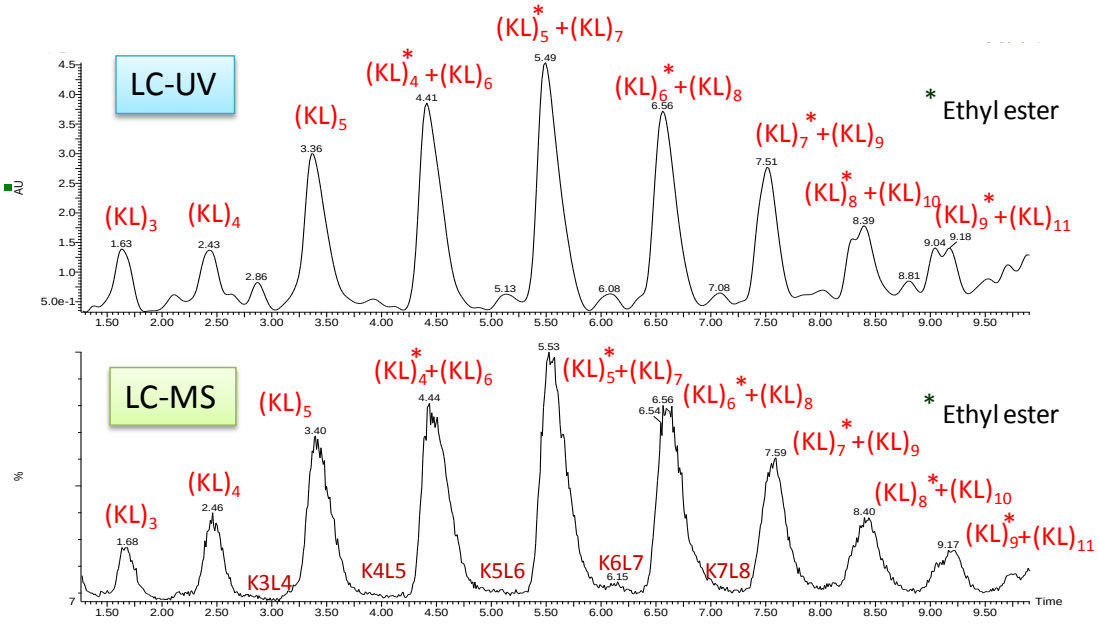


Figure S-2: HPLC-UV-MS chromatograms of alternating peptide $(KL)_x$ synthesized by α-chymotrypsin at pH 8.5 from substrate concentration 12% KL-OEt w/w. By analysis of LC-MS chromatograms at retention times between large peaks, a small fraction of KL peptides with structures $K_{x-1}L_x$ was identified. The extent that this population of peptides corresponds to minor signals observed between major component peaks in LC-UV spectra is currently unknown.

Careful analysis of LC-MS chromatograms at retention times between large peaks shows that α-chymotrypsin-catalyzed oligomerization of KL-OEt results in a small fraction of KL peptides with structures $K_{x-1}L_x$ (Figure S-2). Calculation of DP_{avg} by HPLC-UV-MS is based on areas of assigned peaks in the LC-UV chromatogram. Since constituents such as $(KL)_4$* and $(KL)_6$ are not resolved and the relative contributions of each are unknown, DP_{avg} was determined by assuming all peaks at or after 4.4 min consist exclusively of either $(KL)_x$ ester or $(KL)_x$ free acid. By this approach, DP_{avg} from LC-UV analysis is 5.0 and 6.2, respectively.

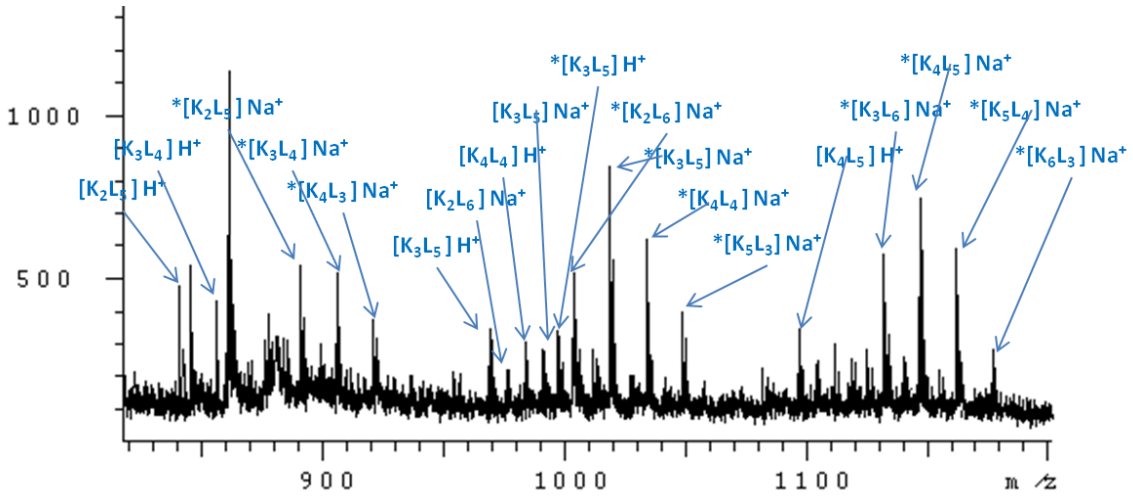


Figure S-3: MALDI-TOF spectra of oligopeptides synthesized from KL-OEt using papain as catalyst at pH 8 from substrate concentration 12% KL-OEt w/w. The MALDI-TOF spectrum shows that synthesized oligopeptidesconsisting of K and L-units deviate significantly from strictly alternating sequences. The term [(K_xL_y)] is an abbreviation for oligopeptides having x-units of Lys and y-units of Leu repeat units. Peaks corresponding to oligopeptides with its ethyl ester group intact at the C-terminus are designated by an asterisk [*]. Values of m/z are ± 1 Da of those expected for the molecular ion peaks.

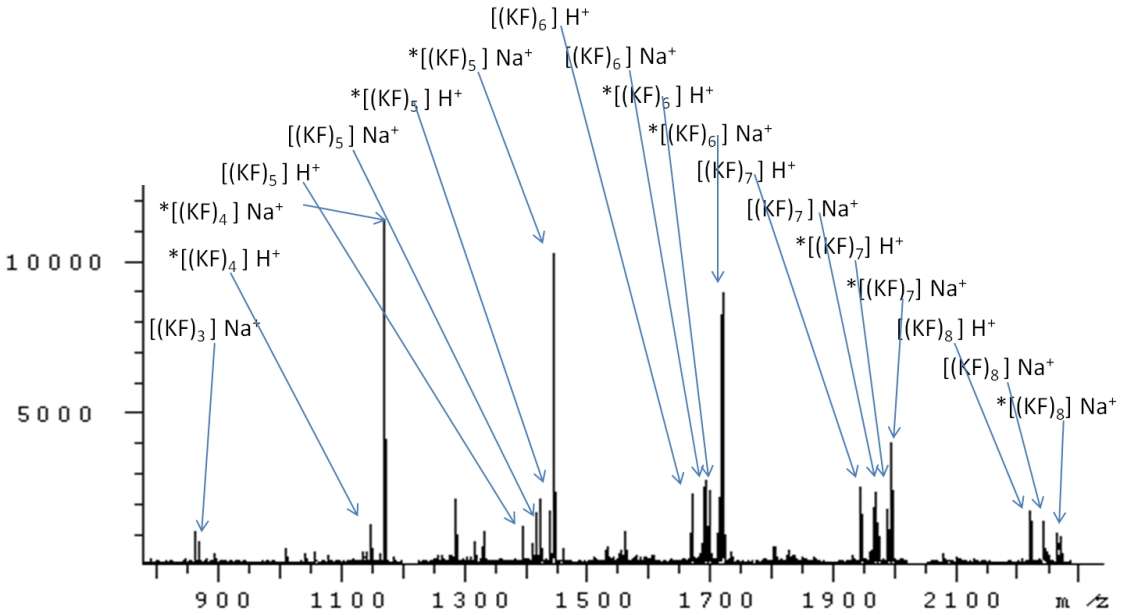


Figure S-4: MALDI-TOF spectra of alternating peptide $(KF)_x$, synthesized by □-chymotrypsin at pH 9 from substrate concentration 12% KF-OEt w/w. $(KF)_x$ is an abbreviation for x units of Lys-Phe dipeptide repeat units. Values of m/z observed are ± 1 Da of those expected for molecular ion peaks. Peaks corresponding to oligopeptides with its ethyl ester group intact at the C-terminus are designated by an asterisk [*]. Values of m/z are ± 1 Da of those expected for the molecular ion peaks.

References

(1) Isidro-Llobet, A.; lvarez, M. A.; Albericio, F. *Chem. Rev.* **2009**, *109*, 2455.

(2) Qin, X.; Xie, W.; Su, Q.; Du, W.; Gross, R. A. *ACS Catalysis* **2011**, *1*, 1022.

(3) Viswanathan, K.; Schofield, M. H.; Teraoka, I.; Gross, R. A. *Green Chemistry* **2012**, *14*, 1020.