Optimization of interstrand interactions enables burn detection with a collagen-mimetic peptide

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Abbreviations Used

ACN	acetonitrile
CD	circular dichroism
CHCA	α-cyano-4-hydroxycinnamic acid
CMP	collagen mimetic peptide
DIC	*N,N′*-diisopropylcarbodiimide
DCM	dichloromethane
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
Fmoc	fluorenylmethyloxycarbonyl
HOBt	hydroxybenzotriazole
MALDI–TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
PBS	phosphate-buffered saline
TEA	triethylamine
*t*Bu	*tert*-butyl
TFA	trifluoroacetic acid
TIS	triisopropylsilane

Computational Evaluation of Interstrand Xaa···Yaa Side-Chain Interactions

Calculations were performed on Intel Xeon 2.33-GHz processors at the Materials and Process Simulation Center at the California Institute of Technology (Pasadena, CA). Computational models built based on the crystal structure of the $(PPG)_{10}$ trimer (PDB entry 1kf6)[1] were supplemented with hydrogen atoms using REDUCE (version 3.03),[2] and the complete model was minimized fully. Minimizations were carried out to a 0.2 (kcal/mol)/Å RMS-force convergence criterion using conjugate gradient minimization on MPSIM.[3] Interatomic forces were described by the DREIDING force-field[4] using CHARMM22 charges[5] and without solvation. Charges on non-canonical amino acids were assigned based on using Mulliken charges obtained through density functional theory calculations at B3LYP/6-311G** level of theory implemented using JAGUAR (version 7.0) from Schrödinger (New York, NY).

Xaa–Yaa interaction energies (*IE*) were calculated on a $(ProProGly)_3$ background. Pro or Pro analogs were installed at the Xaa and Yaa position of neighboring sites on separate strands. Substituents on C^{γ} of each Pro or Pro analog were minimized both in a triple helix (*i.e.*, with a neighbor) and in the absence of other strands (*i.e.*, without a neighbor), and their energies were calculated. The energy of an Xaa···Yaa interaction is defined as the energy of the triple helix hosting the Xaa···Yaa pair relative to the sum of the energies for individual strands making up the triple helix.

General Experimental Procedures

Reagents. Commercial chemicals were of reagent grade or better, and were used without further purification. Natural amino , flpOH, and HOBt were from Chem-Impex International (Wood Dale, IL). FmocflpOH and FmocHyp(*t*Bu)OH were from OmegaChem (Saint-Romuald, Canada). FmocGly-loaded Wang resin was from MilliporeSigma (Burlington, MA). Cyanine5 NHS ester was from Lumiprobe (Hunt Valley, MD). DIC and 4-methylpiperidine were from Oakwood Chemical (Tampa, FL). Anhydrous DMSO, TIS, TFA, and PBS (product P3813) were from Sigma–Aldrich (St. Louis, MO). All other reagents were from Fisher Scientific (Hampton, NH).

Conditions. All procedures were performed at ambient temperature (~23 °C) and pressure (1.0 atm) unless indicated otherwise.

Peptide Synthesis–General

Peptides were synthesized with a Liberty Blue™ Automated Microwave Peptide Synthesizer from CEM (Matthews, NC). All peptides were synthesized following CEM standard methods for both microwave and coupling cycles. Standard solutions of monohydrated HOBt (1 M in DMF), DIC (0.5 M in DMF), 4-methylpiperidine (20% v/v in DMF), and Fmoc-protected amino acids (0.2 M in DMF) were prepared for each synthesis.

Standard Microwave-Assisted Deprotection. The microwave was set to 155 W at 75 °C for 15 s, followed by 30 W at 90 °C for 50 s.

Standard Microwave-Assisted Coupling. The microwave was set to 170 W at 75 °C for 15 s, followed by 30 W at 90 °C for 225 s.

*Standard Coupling Cycl*e. FmocGly-loaded Wang resin (1 equiv) was added to the CEM reaction vessel, and the resin was allowed to swell for 5 min in DMF. The Fmoc group was removed using the standard deprotection solution and the microwave-assisted deprotection methods described above. The resin was then washed (4×), and Fmoc-AA-OH (5 equiv) was added, followed by DIC (20 equiv) and HOBt monohydrate (40 equiv). Standard microwave-assisted coupling was performed with additional Fmoc-protected amino acids, and the resin was washed (2×) and drained. When double-coupling was required, the cycle was repeated without the deprotection step.

Cleavage and Precipitation. After the final deprotection step, the resin was removed from the reaction vessel into a cleavage filter, washed with DCM (4×), and air-dried Crude peptides were then cleaved from the resin using a cleavage cocktail composed of H_2O/TIS/TFA 2.5:2.5:95 for 2 h. Peptide mixtures were then filtered and precipitated in ice-cold diethyl ether (10×). The peptides were collected by centrifugation, the supernatants were decanted, and the solid peptide was dissolved in 5 mL of H_2O/ACN 70:30. The solutions were frozen and lyophilized using a FreeZone benchtop instrument from Labconco (Kansas City, MO). The crude peptide mixture was then subjected to purification.

Purification. The crude peptide products were purified by preparative reverse-phase HPLC using a VP 250/21 Nucleosil 100-5 C18 column from Macherey–Nagel (Bethlehem, PA) and a 1260 Infinity II instrument from Agilent Technologies (Santa Clara, CA). Crude products were dissolved in the minimum amount of ACN and eluted with a linear gradient of 5–80% ACN/H_2O containing TFA (0.1% v/v). After reviewing the initial chromatogram, the method was updated, if necessary. Chromatography fractions were analyzed by MALDI–TOF MS using a microflex LRF instrument and a CHCA matrix from Bruker (Billerica, MA). Fractions containing purified peptide were pooled and lyophilized, and analyzed by reverse-phase HPLC using an EC 250/4.6 Nucleosil 100-5 C18 column from Macherey–Nagel and a 1260 Infinity II instrument from Agilent Technologies.

CD Spectroscopy. Peptides were dried under vacuum for ≥48 h before being weighed and dissolved to 0.8 mM in 50 mM acetic acid (pH 3.0). The resulting solutions were heated to 65 °C and cooled to 4 °C at a rate of 1 °C every 5 min. The solution was then incubated at ≤4 °C for ≥24 h before its CD spectrum was acquired with a Model J-1500 spectrometer from JASCO (Easton, MD) at the MIT Biophysics Instrumentation Facility. Spectra were measured with a band-pass of 1 nm. The signal was averaged for 3 s during the wavelength scan. At each temperature,

solutions were allowed to equilibrate for a minimum of 8 min before data acquisition. Values of T_m were determined by fitting the molar ellipticity at 225 nm to a two-state model.

Peptide Synthesis–Specific

(ProProGly)$_7$. (ProProGly)$_7$ was synthesized by a non-interrupted continuous method. After deprotection of FmocGly-loaded Wang resin, Fmoc-protected amino acids were coupled through a single standard coupling cycle until completion. Following deprotection, the peptide was cleaved from the resin and precipitated to afford a crude peptide product. The crude product was purified with preparative reverse-phase HPLC, and chromatography fractions were analyzed by MALDI–TOF MS in positive-ion mode. Fractions containing pure material were pooled and lyophilized.

(flpHypGly)$_7$, (HypflpGly)$_7$, Gly-(SerGly)$_2$-(flpHypGly)$_7$, and Gly-(SerGly)$_2$-(HypflpGly)$_7$. CMPs were synthesized by a non-interrupted continuous method. After deprotection of FmocGly-loaded Wang resin, Fmoc-protected amino acids were coupled by either a single or a double standard coupling cycle until completion. The low nucleophilicity of flp and Hyp required a double standard coupling cycle. Following deprotection, the peptide was cleaved from the resin and precipitated to afford a crude peptide product. The crude product was purified with preparative reverse-phase HPLC, and chromatography fractions were analyzed by MALDI–TOF MS in positive-ion mode. Fractions containing pure material were pooled and lyophilized.

Cy5–Gly-(SerGly)$_2$-(flpHypGly)$_7$ and Cy5–Gly-(SerGly)$_2$-(HypflpGly)$_7$ Conjugates. The peptide was dissolved in DMSO containing TEA (4 equiv), and the resulting solution was allowed to stir for 15 min. Cy5-NHS ester (2 equiv) was added to the mixture, and the resulting solution was allowed to react overnight in the dark. The reaction mixture was then diluted with water (5 equiv), frozen, and lyophilized. The peptide conjugate was purified with preparative reverse-phase HPLC, and chromatography fractions were analyzed by MALDI–TOF MS in positive-ion mode. Fractions containing pure material were pooled, lyophilized, and stored in the dark.

Cytotoxicity Assays

Human fibroblasts (strain HTB-102) were from ATCC (Manasses, VA). Primary human epidermal keratinocytes were a generous gift from Dr. Jean Christopher Chamcheu and Professor Hasan Mukhtar (University of Wisconsin–Madison). Cells were treated for 24, 48, or 72 h with HflpOH (0.1 μM→1 mM). Cell viability was measured in triplicate with a tetrazolium dye-based assay for metabolic activity[6] (CellTiter 96® AQueous One Solution Cell Proliferation Assay) from Promega (Madison, WI).

Rat-Tail Tendon Extraction and Imaging

Rat tails were obtained from the Animal Surgery Laboratory of the Research Animal Resource Center at the University of Wisconsin–Madison. The tails were excised postmortem and stored at –80 °C. To extract a tendon, a tail was rolled between the hand and a hard surface (table top) to loosen the tendons within the tail. Two transverse cuts were made spaced at the desired tendon length using a bone-cleaving knife to separate the tail. At this stage, the skin could be removed for visualizing the packed tendons inside and avoiding the skin cut debris. The tendon was exposed from the distal end using a pair of tweezers. Three identical tendons were used for burning and imaging. The tendons were wounded by burns using a hot soldering iron set at 350 °C. A glass microscope slide was placed atop the tendon to prevent charring from direct metal contact. After creating the burn wound, 100 μL of PBS or PBS containing CMP (100 μM) or CI (100 μM) was

applied to the burn site. The tendons were incubated in the dark for 1 h and then washed three times with PBS and retained in PBS to prevent dehydration.

Tendons were imaged using an upright multiphoton laser scanning microscope (Ultima IV multiphoton Microscope System, Bruker Nano Surface Division, Middleton, WI) equipped with a tunable ultrafast pulsed laser (Insight DeepSee, Spectra-Physics, Santa Clara, CA). The best multiphoton excitation efficiency of the Cy5 dye using Insight laser was determined to be at 820 nm by measuring a multiphoton excitation spectrum in the excitation range from 800 to 1200 nm. The Cy5 emission was collected using a 690/50 nm emission bandpass filter (AT690/50m, Chroma Technology Corporation, VT). Second harmonic Generation (SHG) imaging of collagen was collected using a 445/40 nm bandpass filter (FF01-445/40-25, Semrock, Rochester, NY) using illumination at 890 nm. Sequentially, both channels (CY5, SHG) imaged the entire tendon using a 10× objective (Nikon, 0.75 N.A.) tiling the image area using an automated mechanical xy stage on the multiphoton microscope. The images were stitched, and average intensities within the damaged and undamaged regions of interest were determined with ImageJ software (NIH).[7] Experiments were performed in duplicate.

References

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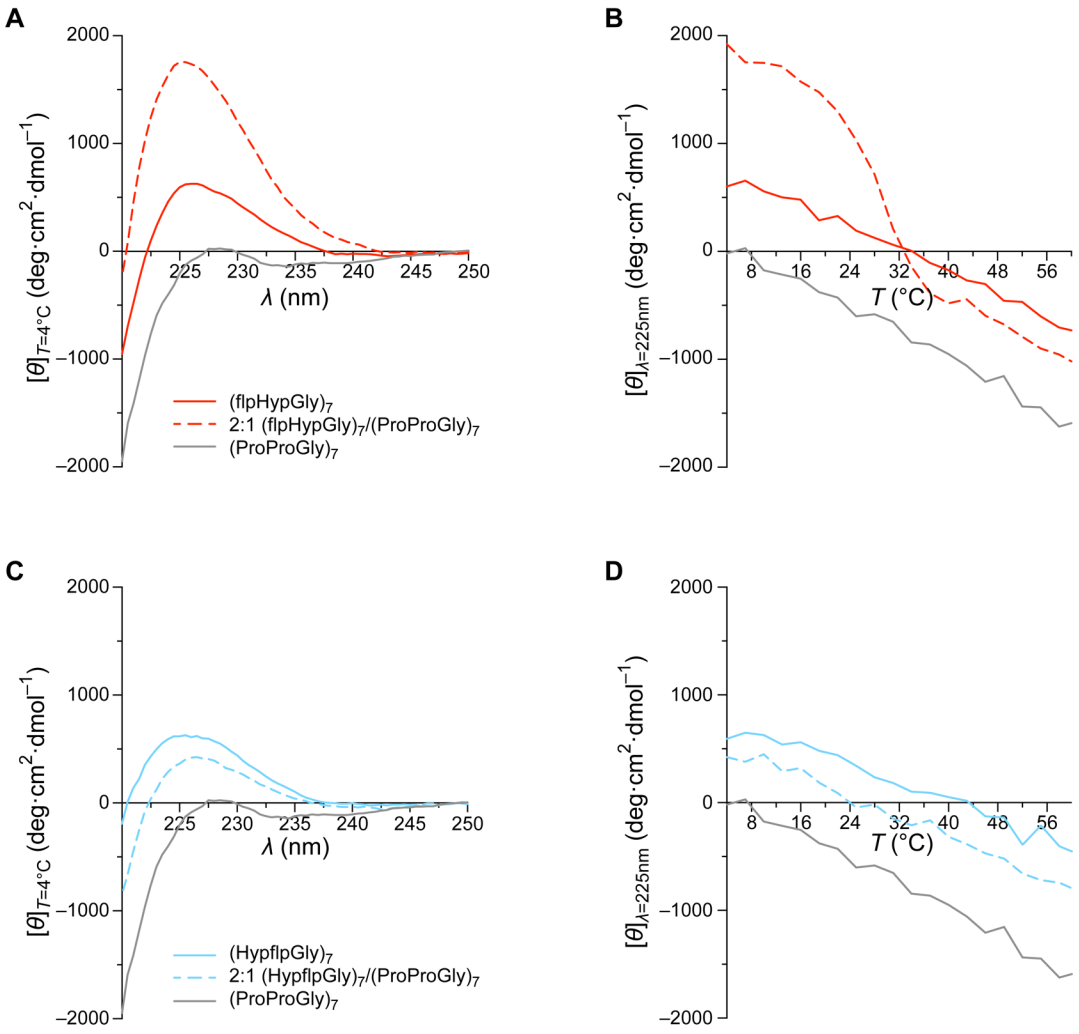


Figure S1. Analyses of CMPs with circular dichroism spectroscopy. Each solution contained 0.8 mM CMP (or a mixture) in 50 mM acetic acid. Data for $(flpHypGly)_7$, $(HypflpGly)_7$, and $(ProProGly)_7$, but not the 2:1 mixtures, are identical to those in Figure 3. (A) Spectra of $(flpHypGly)_7$, $(ProProGly)_7$, and a 2:1 mixture at 4 °C. (B) Graph showing the effect of temperature on the molar ellipticity at 225 nm of $(flpHypGly)_7$, $(ProProGly)_7$, and a 2:1 mixture. The mixture had $T_m = 28$ °C. (C) Spectra of $(HypflpGly)_7$, $(ProProGly)_7$, and a 2:1 mixture at 4 °C. and its mixture at 4 °C. (D) Graph showing the effect of temperature on the molar ellipticity at 225 nm of $(HypflpGly)_7$, $(ProProGly)_7$, and a 2:1 mixture.

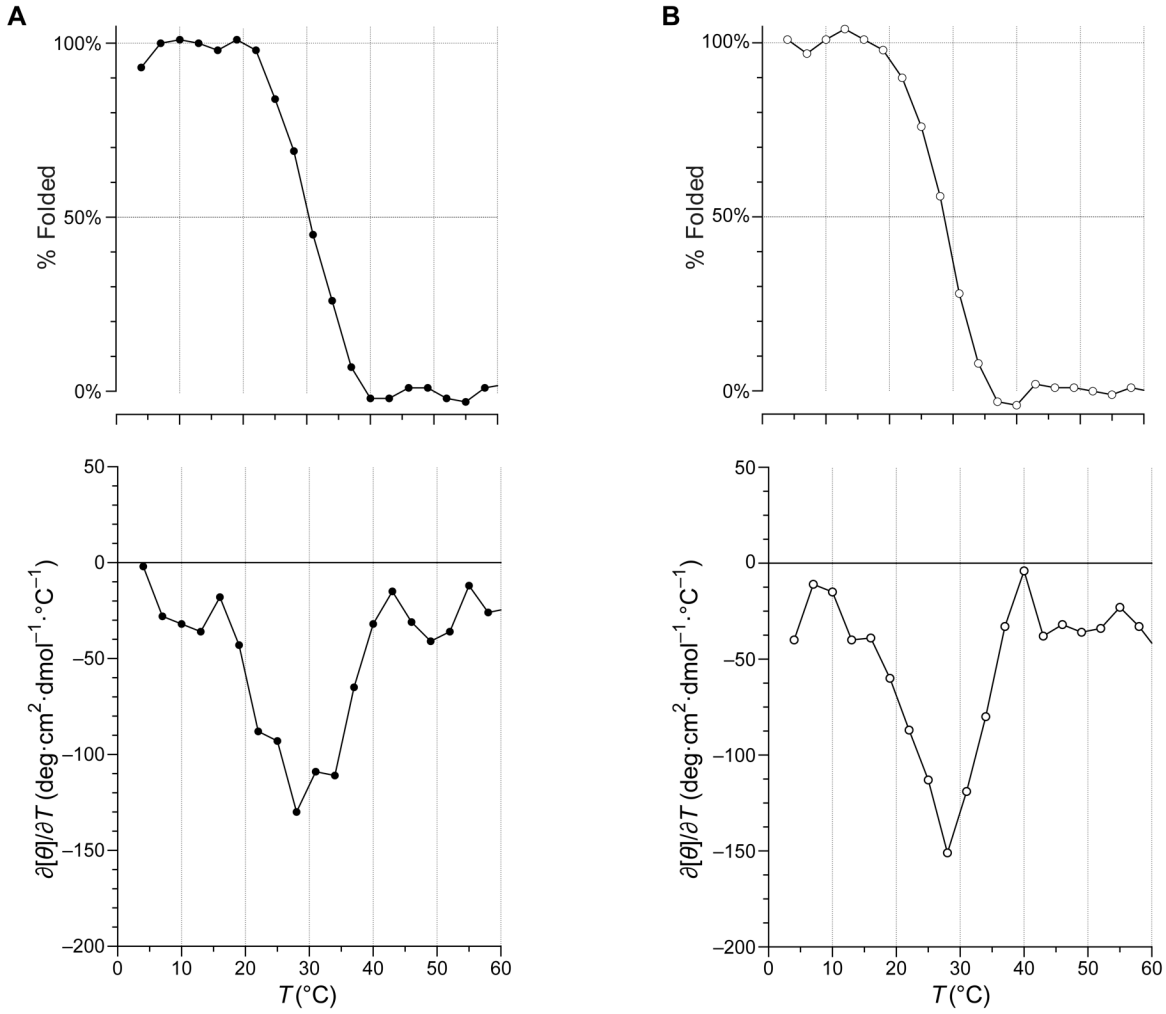


Figure S2. Transformed circular dichroism data for the thermal denaturation of CMP mixtures based on two-state model for triple-helix unfolding. (A) Transformed data of 1:2 $(flpHypGly)_7/(ProProGly)_7$ (Figure 3B); T_m = 28 °C (B) Transformed data of 2:1 $(flpHypGly)_7/(ProProGly)_7$ (Figure S1B); T_m = 28 °C.

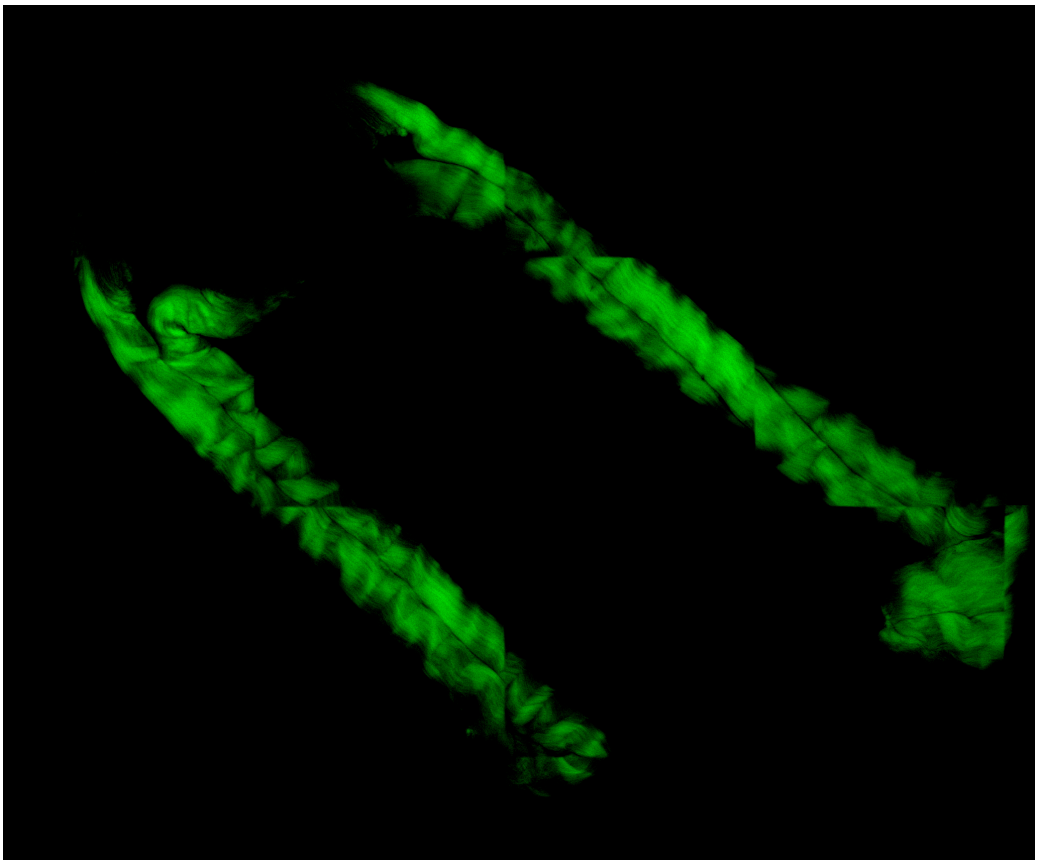
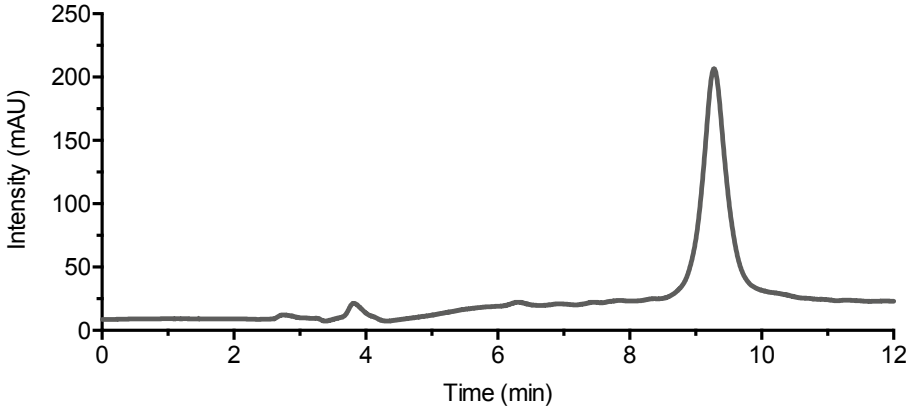


Figure S3. Second Harmonic Generation (SHG) channel of collagen fibrils. Scale bar, 500 μm.

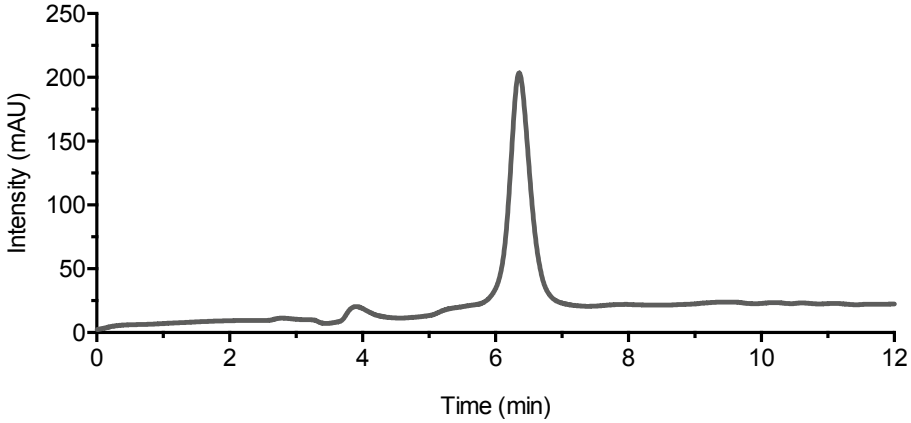
Analytical HPLC Traces

Gradient: 5–70% acetonitrile/H_2O containing TFA (0.1% v/v) over 12 min
Note that traces for mixtures are the summation of those from component peptides.

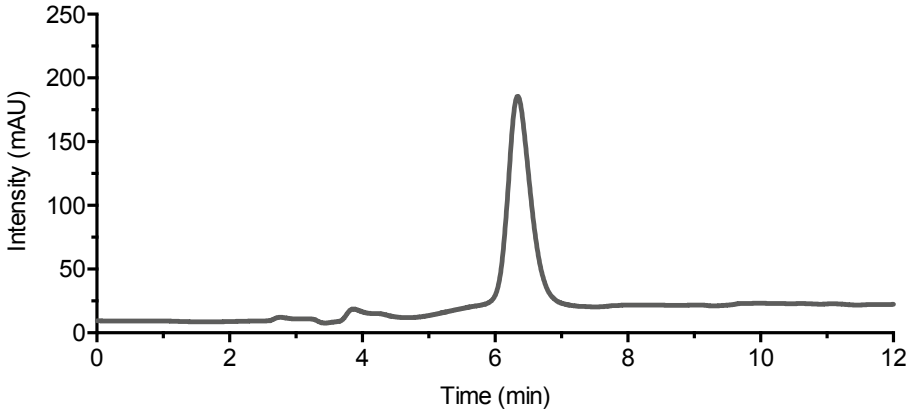
(ProProGly)$_7$



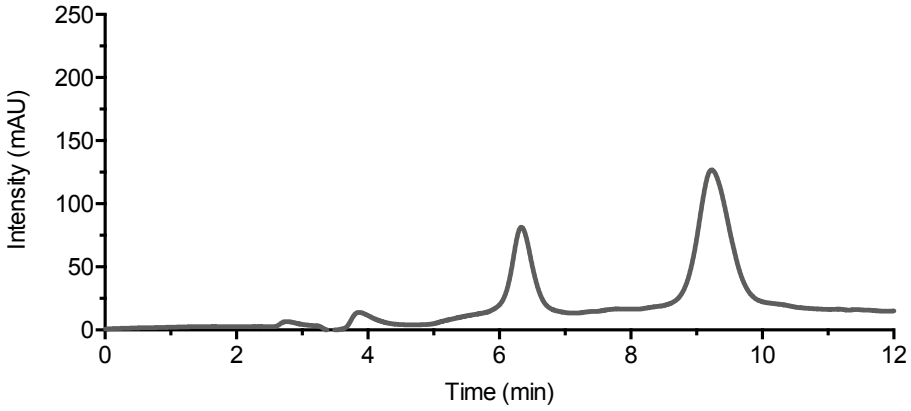
(flpHypGly)$_7$



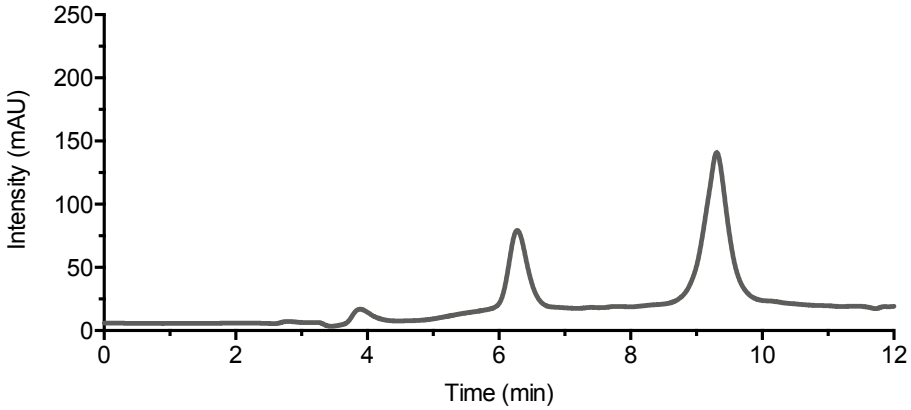
(HypflpGly)$_7$



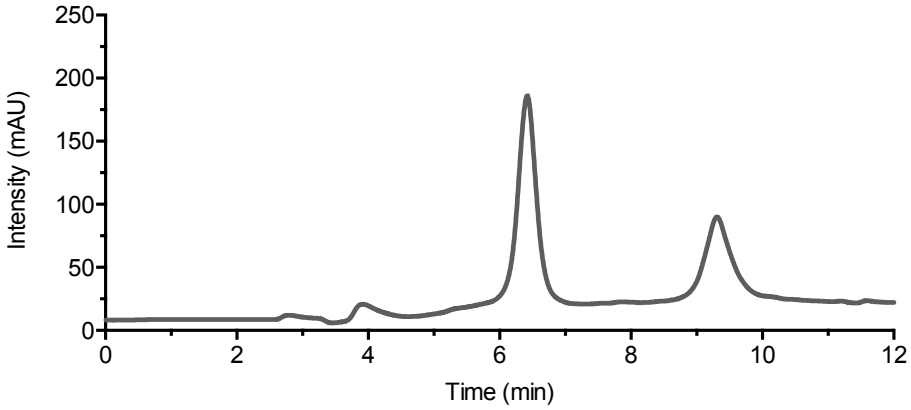
1:2 (flpHypGly)$_7$/(ProProGly)$_7$



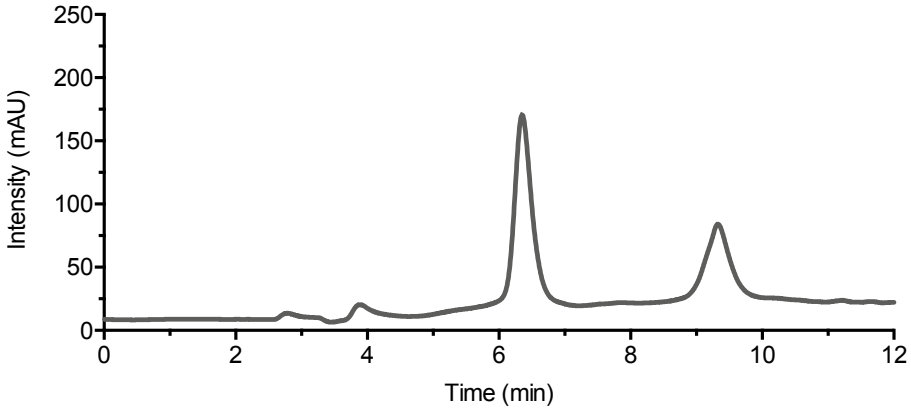
1:2 (HypflpGly)$_7$/(ProProGly)$_7$



2:1 (flpHypGly)$_7$/(ProProGly)$_7$



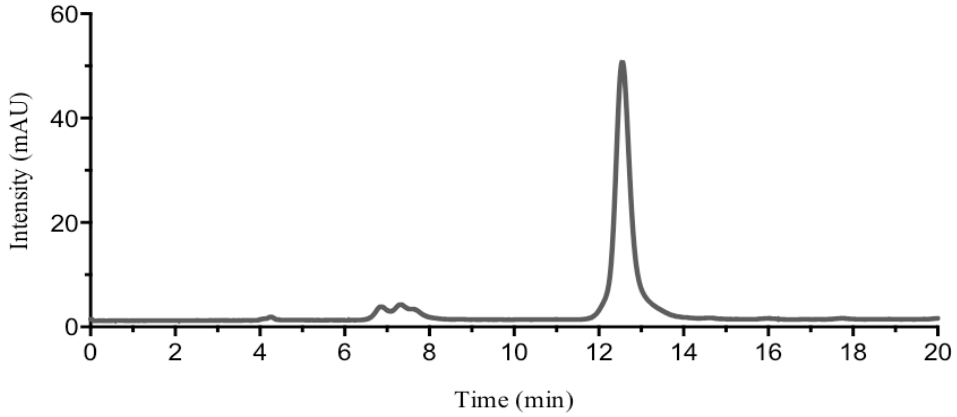
2:1 $(HypflpGly)_7/(ProProGly)_7$



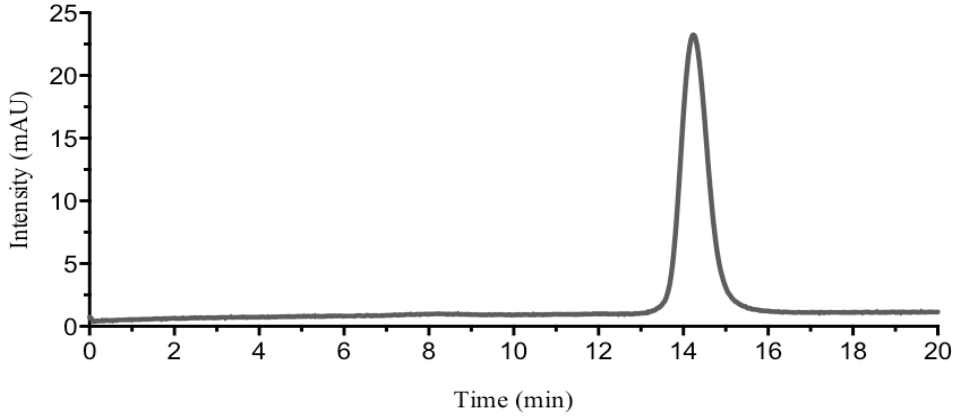
Fluorescently Labeled CMPs

Gradient: 35–42% acetonitrile/H_2O containing TFA (0.1% v/v) over 20 min

^{Cy5}CMP



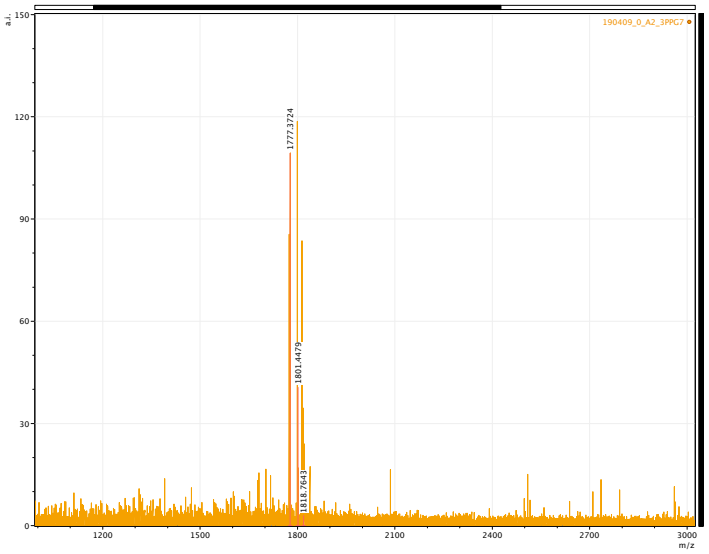
^{Cy5}Cl



MALDI–TOF Mass Spectra

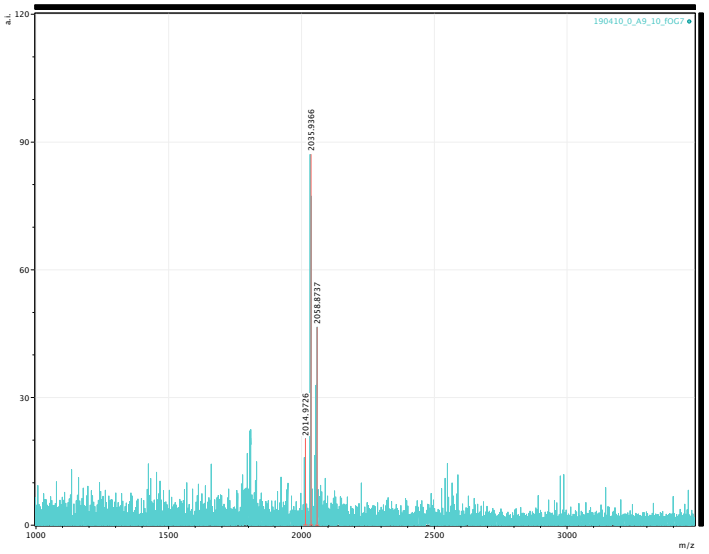
$(ProProGly)_7$

$[M + H]^+$ (DA) calculated, 1777.02; found, 1777.87
$[M + Na]^+$ (DA) calculated, 1800.01; found: 1801.47



$(flpHypGly)_7$

$[M + H]^+$ (DA) calculated, 2014.94; found, 2014.97
$[M + Na]^+$ (DA) calculated, 2036.92; found, 2035.94



(HypfIpGly)$_7$

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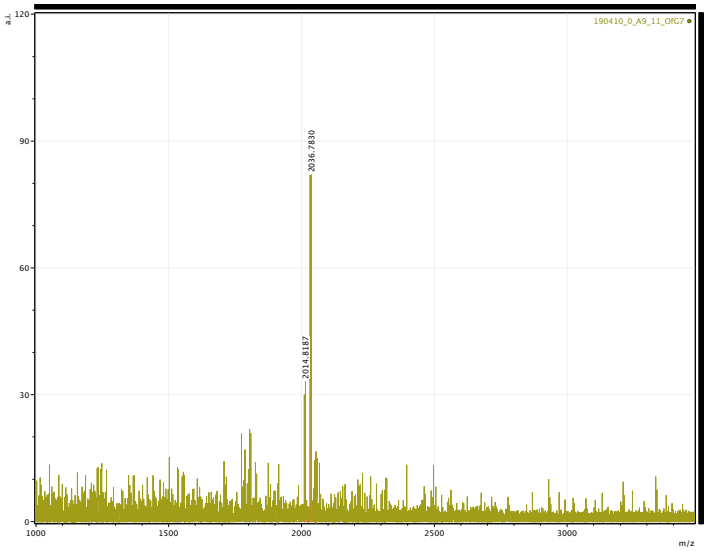
$[M + Na]^+$ (DA) calculated, 2036.92; found, 2036.78



^{Cy5}Cl

$[M + H]^+$ (DA) calculated, 2825.92; found, 2824.78