Electronic Supplementary Information

Insertion of Pro-Hyp-Gly provides 2 kcal/mol stability but attenuates the specific assembly of ABC heterotrimeric collagen triple helices

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Figures

Tables

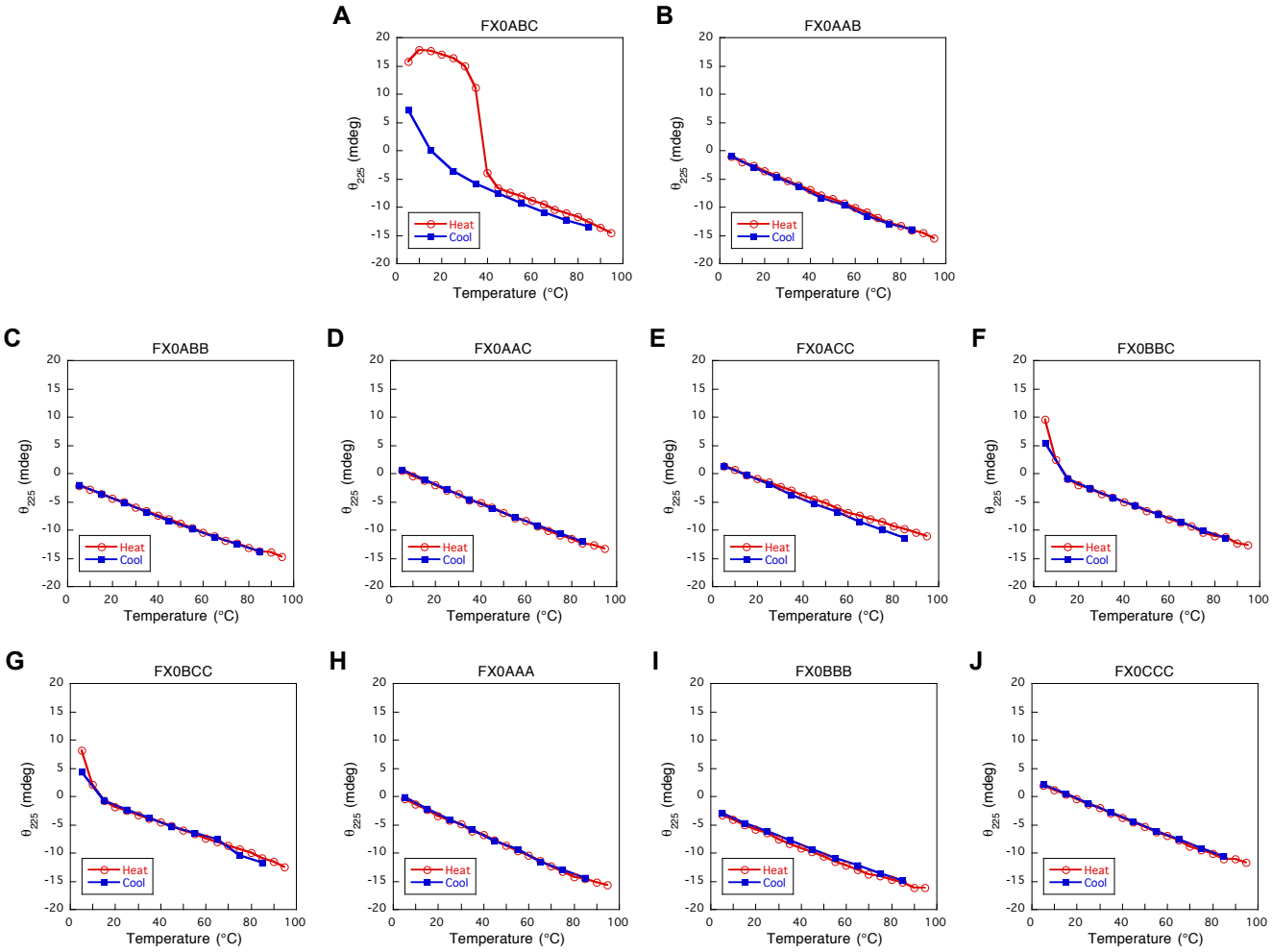

Fig. S1 Preliminary thermal unfolding and folding assessment monitored by circular dichroism spectroscopy at 225 nm with 15 minute equilibration at each temperature with a total peptide concentration of 200 μM in 10 mM pH 7 phosphate buffer for the combinations FX0ABC (A), FX0AAB (B), FX0ABB (C), FX0AAC (D), FX0ACC (E), FX0BBC (F), FX0BCC (G), FX0AAA (H), FX0BBB (I), FX0CCC (J). The heating unfolding data are shown in red and the cooling refolding data are shown in blue.

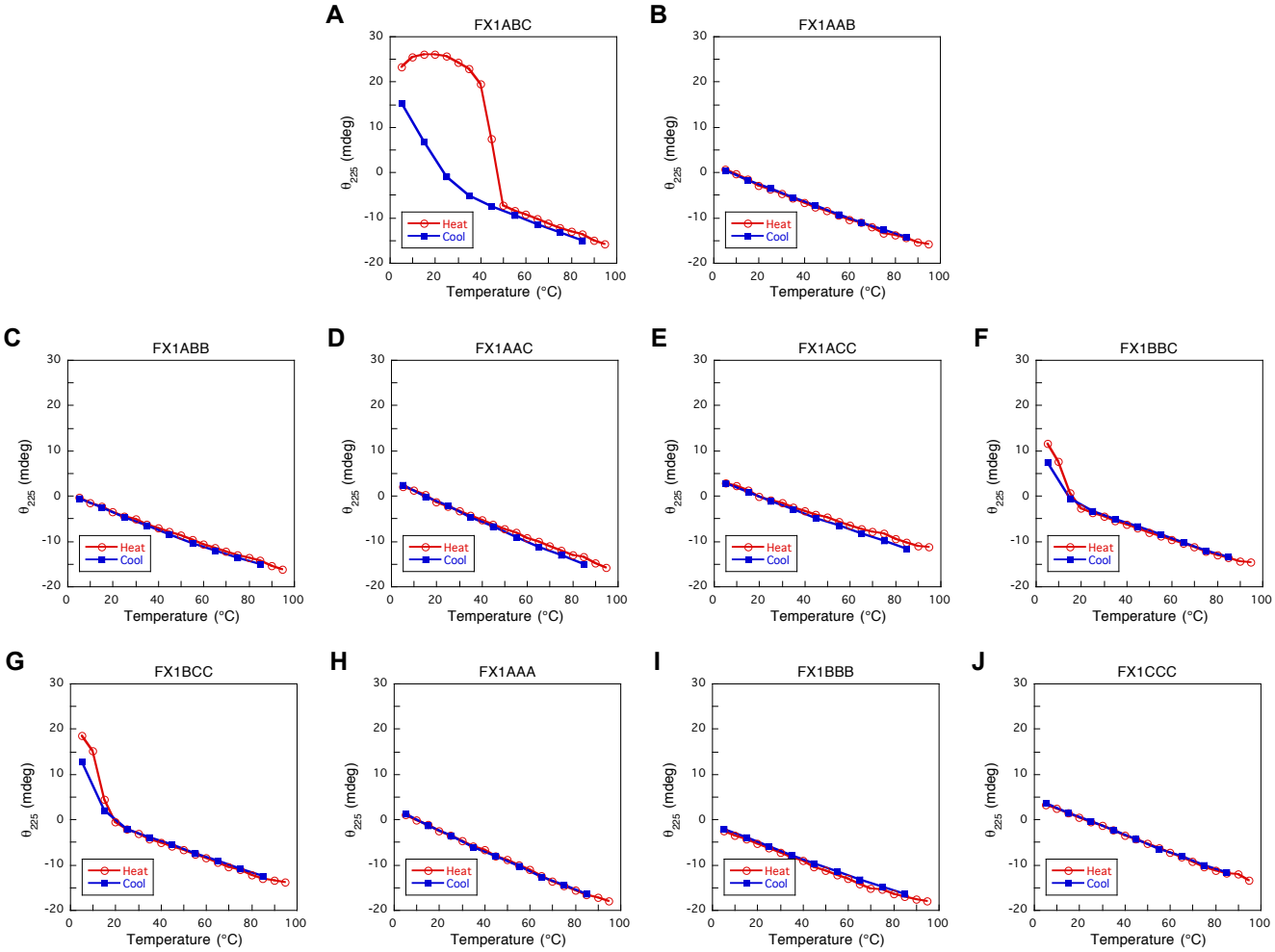

Fig. S2 Preliminary thermal unfolding and folding assessment monitored by circular dichroism spectroscopy at 225 nm with 15 minute equilibration at each temperature with a total peptide concentration of 200 μM in 10 mM pH 7 phosphate buffer for the combinations FX1ABC (A), FX1AAB (B), FX1ABB (C), FX1AAC (D), FX1ACC (E), FX1BBC (F), FX1BCC (G), FX1AAA (H), FX1BBB (I), FX1CCC (J). The heating unfolding data are shown in red and the cooling refolding data are shown in blue.

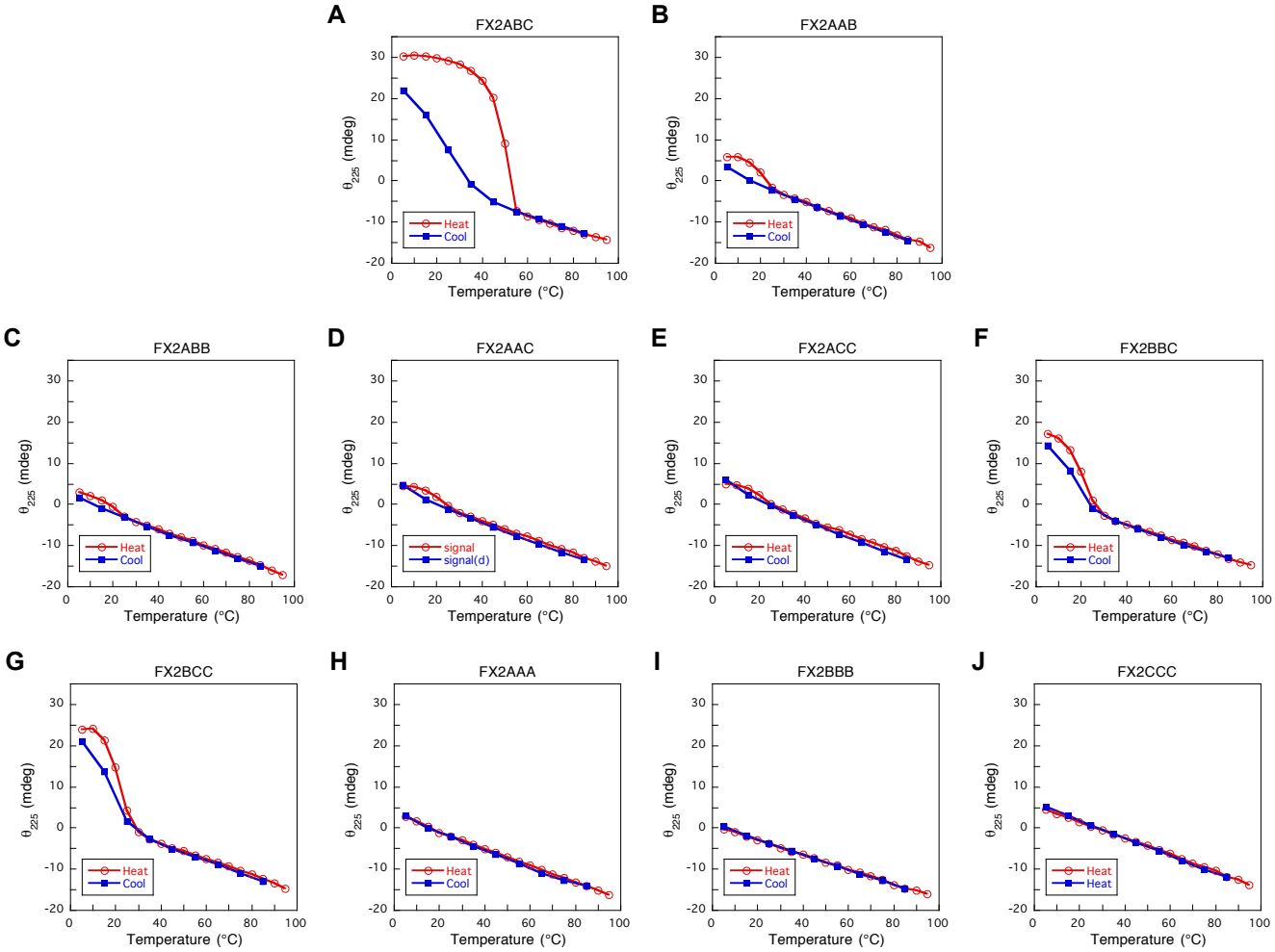

Fig. S3 Preliminary thermal unfolding and folding assessment monitored by circular dichroism spectroscopy at 225 nm with 15 minute equilibration at each temperature with a total peptide concentration of 200 μM in 10 mM pH 7 phosphate buffer for the combinations FX2ABC (A), FX2AAB (B), FX2ABB (C), FX2AAC (D), FX2ACC (E), FX2BBC (F), FX2BCC (G), FX2AAA (H), FX2BBB (I), FX2CCC (J). The heating unfolding data are shown in red and the cooling refolding data are shown in blue.

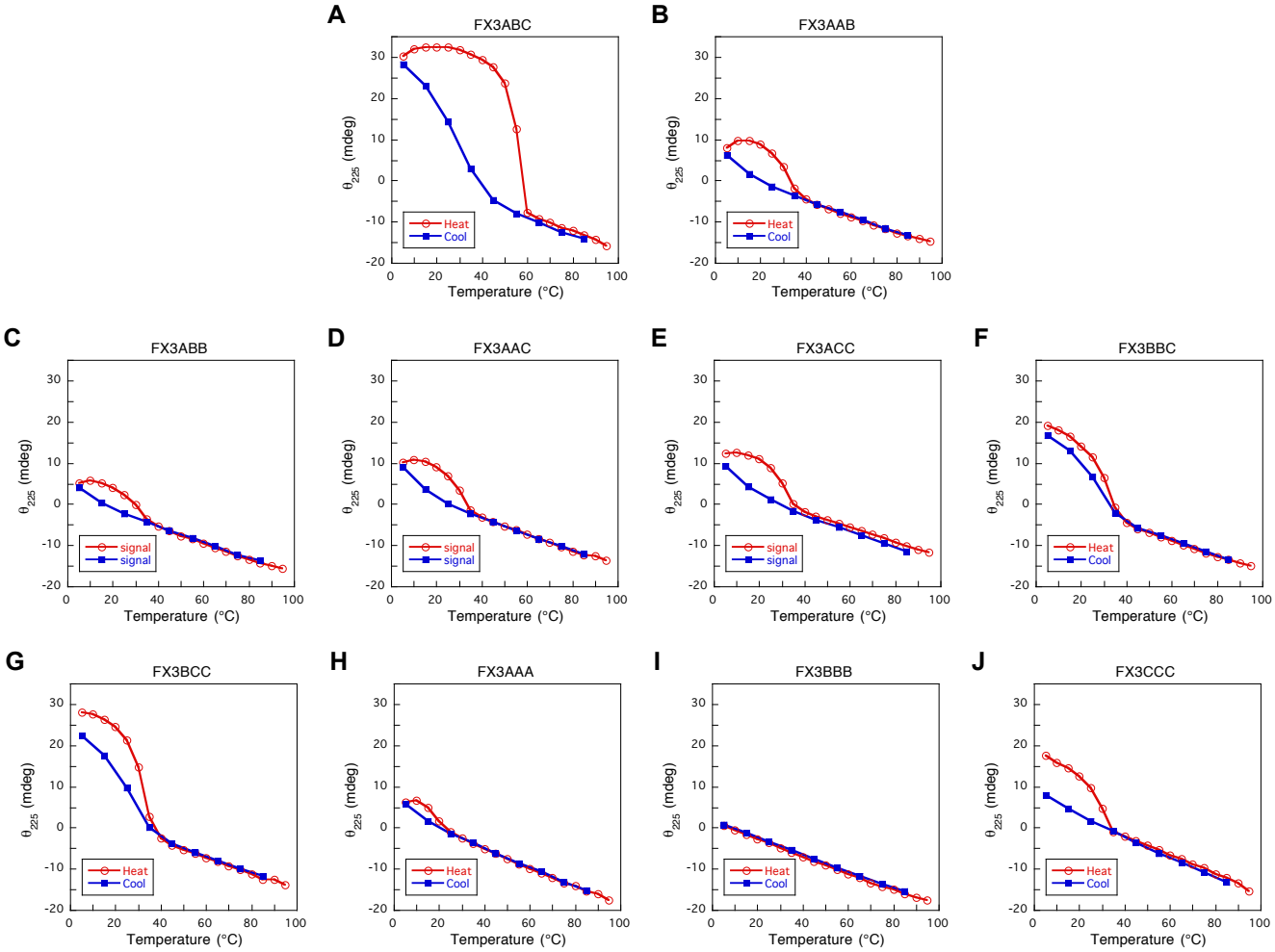

Fig. S4 Preliminary thermal unfolding and folding assessment monitored by circular dichroism spectroscopy at 225 nm with 15 minute equilibration at each temperature with a total peptide concentration of 200 μM in 10 mM pH 7 phosphate buffer for the combinations FX3ABC (A), FX3AAB (B), FX3ABB (C), FX3AAC (D), FX3ACC (E), FX3BBC (F), FX3BCC (G), FX3AAA (H), FX3BBB (I), FX3CCC (J). The heating unfolding data are shown in red and the cooling folding data are shown in blue.

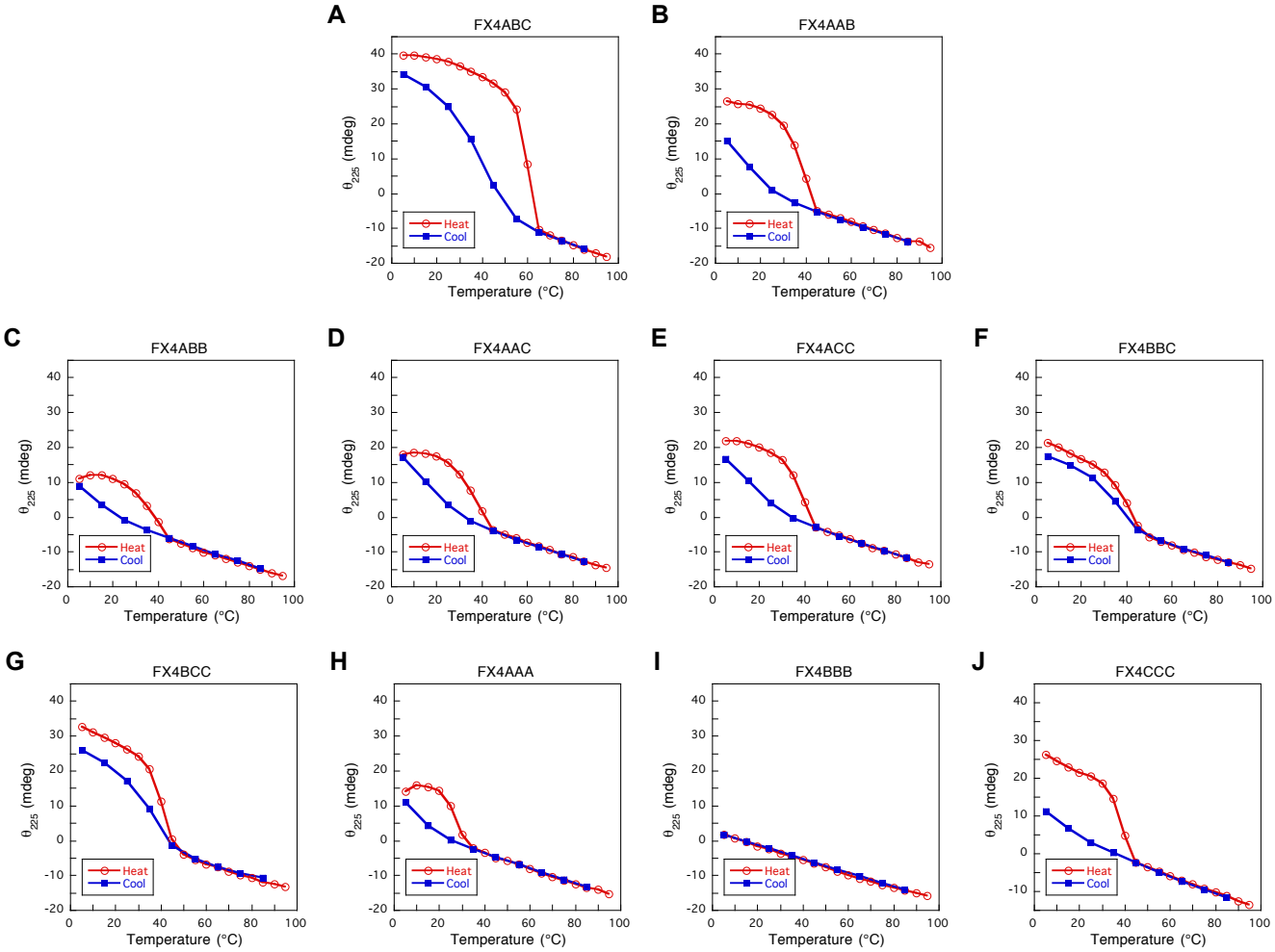

Fig. S5 Preliminary thermal unfolding and folding assessment monitored by circular dichroism spectroscopy at 225 nm with 15 minute equilibration at each temperature with a total peptide concentration of 200 μM in 10 mM pH 7 phosphate buffer for the combinations FX4ABC (A), FX4AAB (B), FX4ABB (C), FX4AAC (D), FX4ACC (E), FX4BBC (F), FX4BCC (G), FX4AAA (H), FX4BBB (I), FX4CCC (J). The heating unfolding data are shown in red and the cooling refolding data are shown in blue.

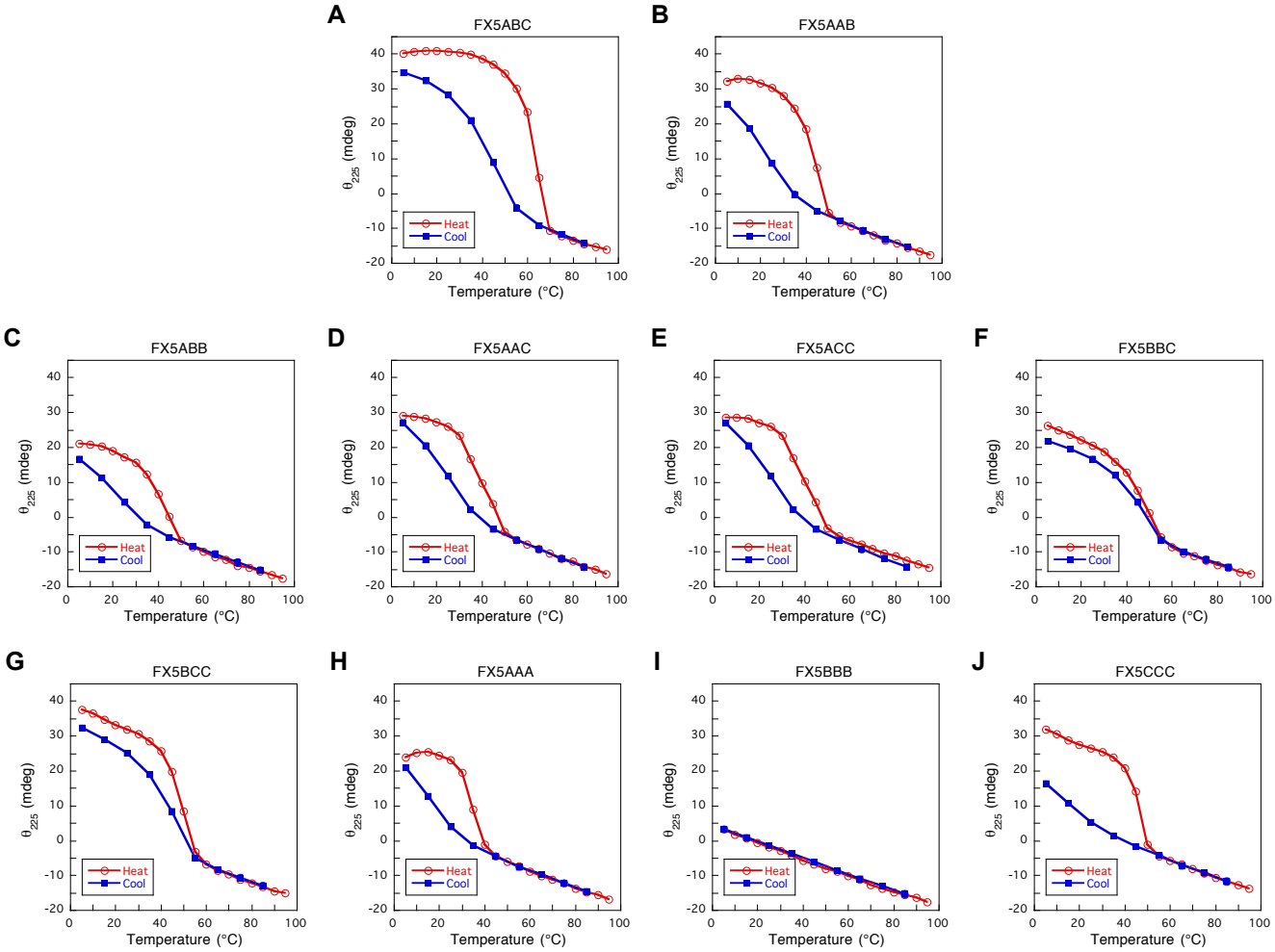

Fig. S6 Preliminary thermal unfolding and folding assessment monitored by circular dichroism spectroscopy at 225 nm with 15 minute equilibration at each temperature with a total peptide concentration of 200 μM in 10 mM pH 7 phosphate buffer for the combinations FX5ABC (A), FX5AAB (B), FX5ABB (C), FX5AAC (D), FX5ACC (E), FX5BBC (F), FX5BCC (G), FX5AAA (H), FX5BBB (I), FX5CCC (J). The heating unfolding data are shown in red and the cooling refolding data are shown in blue.

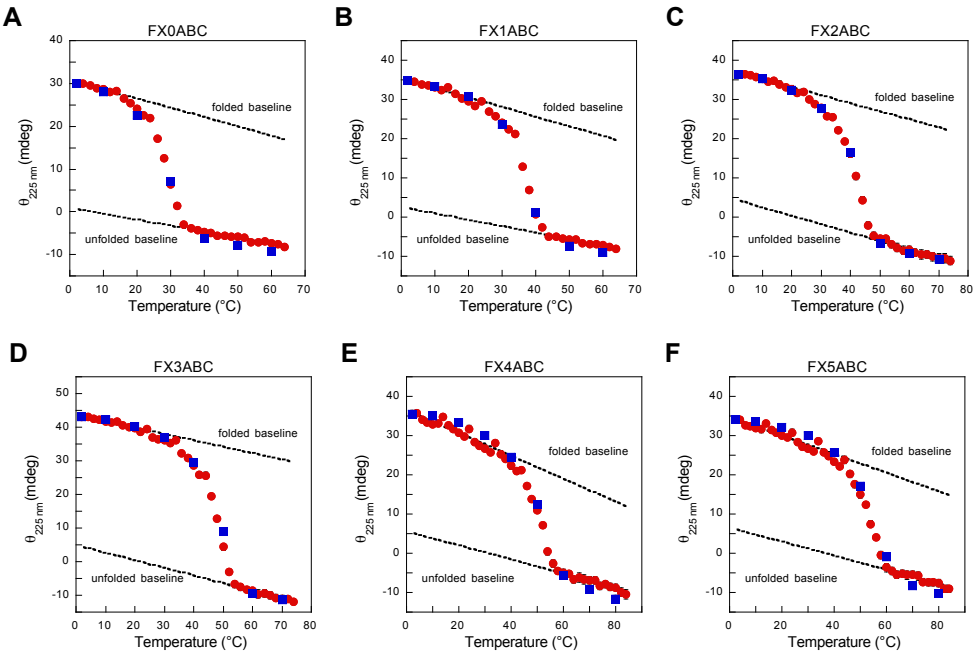

Fig. S7 Thermal unfolding (filled red circles) and folding (blue squares) data acquired with full equilibration at each temperature monitored by circular dichroism spectroscopy at 225 nm with a total peptide concentration of 200 μM in 10 mM pH 7 phosphate buffer for the combinations FX0ABC (A), FX1ABC (B), FX2ABC (C), FX3ABC (D), FX4ABC (E), FX5ABC (F). The pre-transition folded baseline and post-transition unfolded baseline were obtained by linear extrapolation.

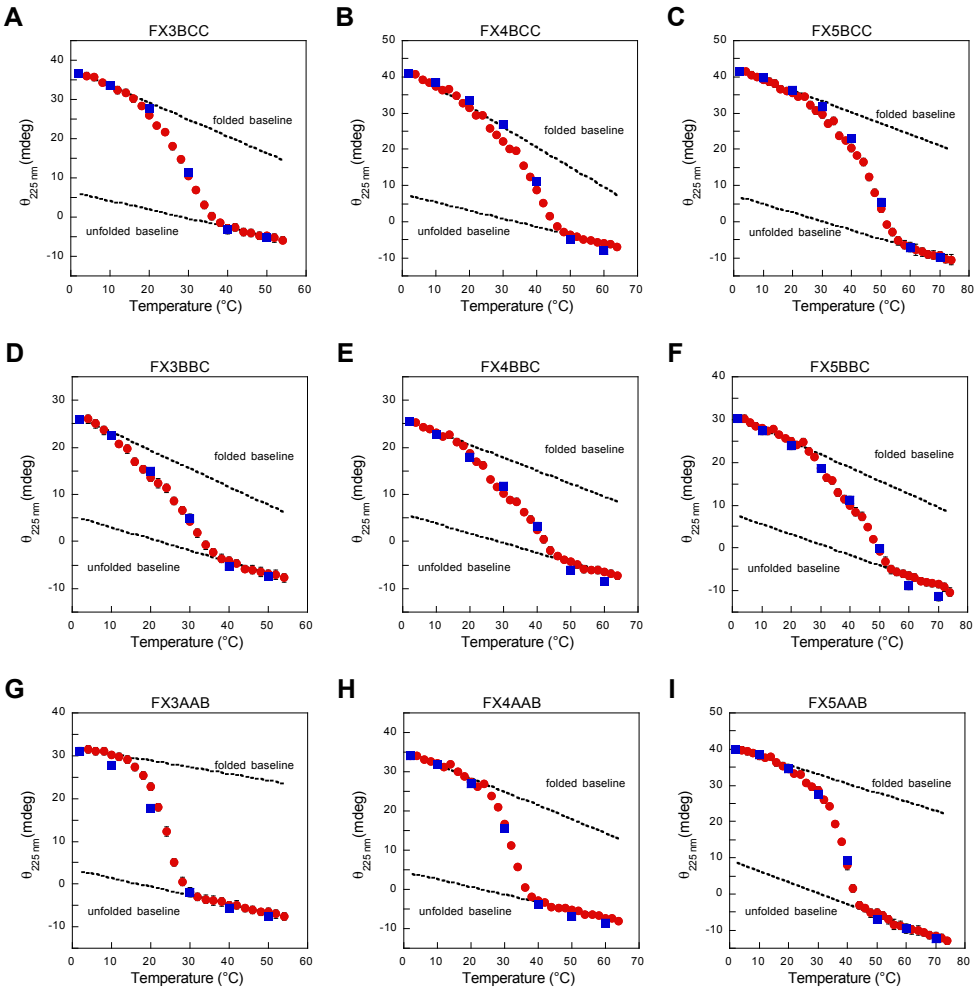

Fig. S8 Thermal unfolding (filled red circles) and folding (blue squares) data acquired with full equilibration at each temperature monitored by circular dichroism spectroscopy at 225 nm with a total peptide concentration of 200 μM in 10 mM pH 7 phosphate buffer for the combinations FX3BCC (A), FX4BCC (B), FX5BCC (C), FX3BBC (D), FX4BBC (E), FX5BBC (F), FX3AAB (G), FX4AAB (H), FX5AAB (I). The pre-transition folded baseline and post-transition unfolded baseline were obtained by linear extrapolation.

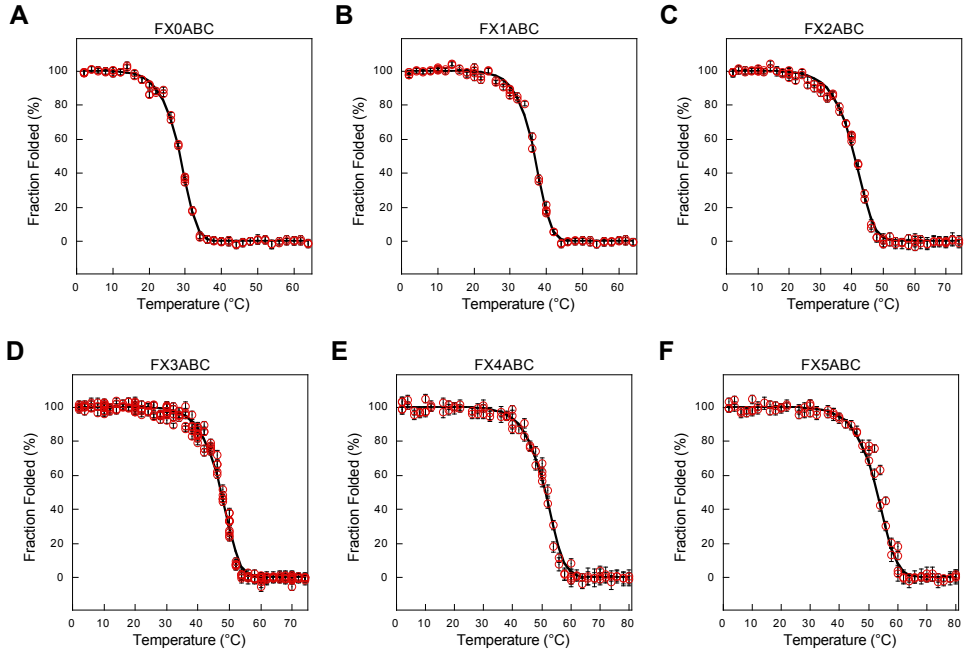

Fig. S9 Fraction folded data plotted against temperature and the corresponding curve fit for the combinations FX0ABC (A), FX1ABC (B), FX2ABC (C), FX3ABC (D), FX4ABC (E), FX5ABC (F). The data are shown as red circles and the curve fits are shown as solid black lines. Data from at least two folding and two unfolding experiments are included.

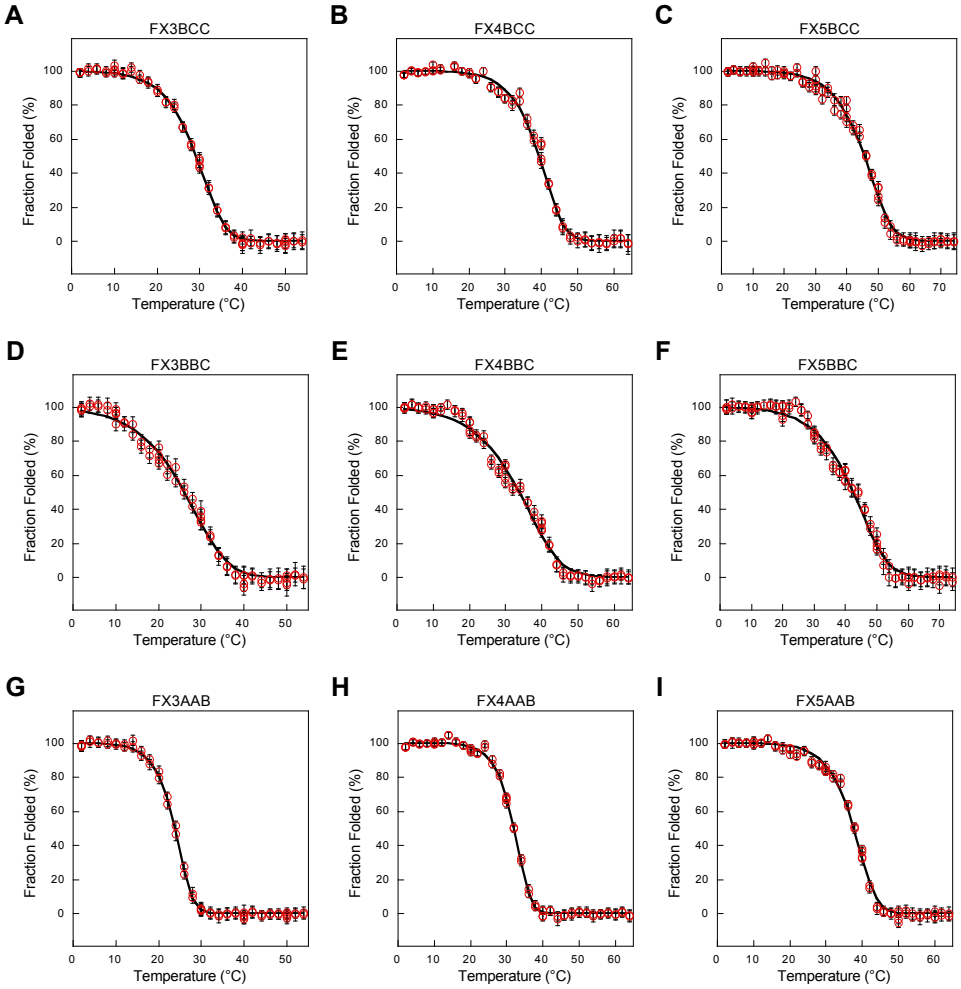

Fig. S10 Fraction folded data plotted against temperature and the corresponding curve fit for the combinations FX3BCC (A), FX4BCC (B), FX5BCC (C), FX3BBC (D), FX4BBC (E), FX5BBC (F), FX3AAB (G), FX4AAB (H), FX5AAB (I). The data are shown as red circles and the curve fits are shown as solid black lines. Data from at least two folding and two unfolding experiments are included.

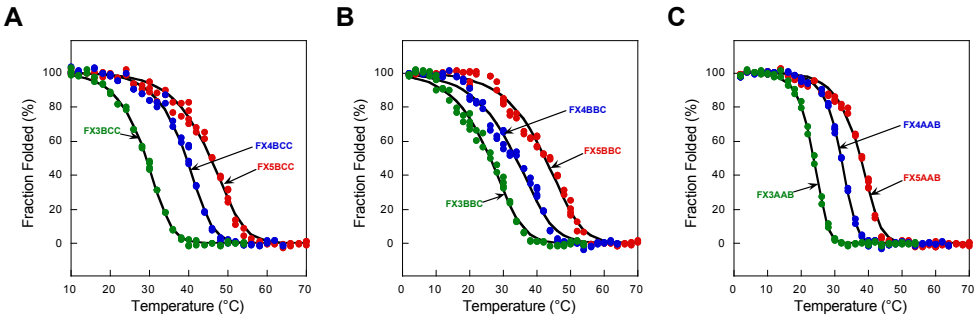

Fig. S11 The temperature dependent fraction folded and the corresponding curve fits for the combinations FX*n*BCC (A), FX*n*BBC (B), and FX*n*AAB (C). The data points are shown as color circles, and the curve fits are shown as solid black lines.

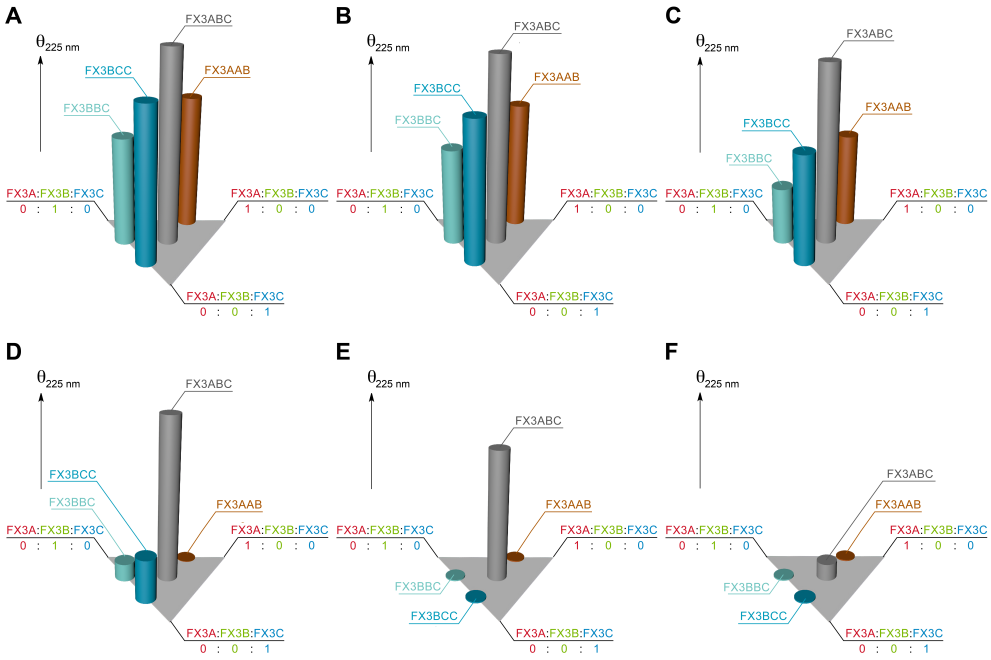

Fig. S12 The three-component Job plots involving peptides FX3A, FX3B, and FX3C at 4°C (A), 10°C (B), 20°C (C), 30°C (D), 40°C (E), and 50°C (F).

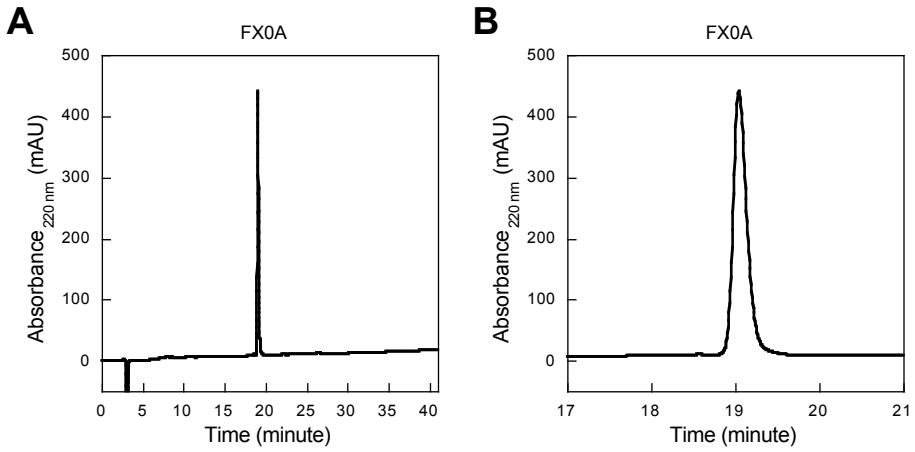

Fig. S13 The analytical HPLC chromatogram of purified FX0A peptide (A) and the zoomed in view of the desired peak (B).

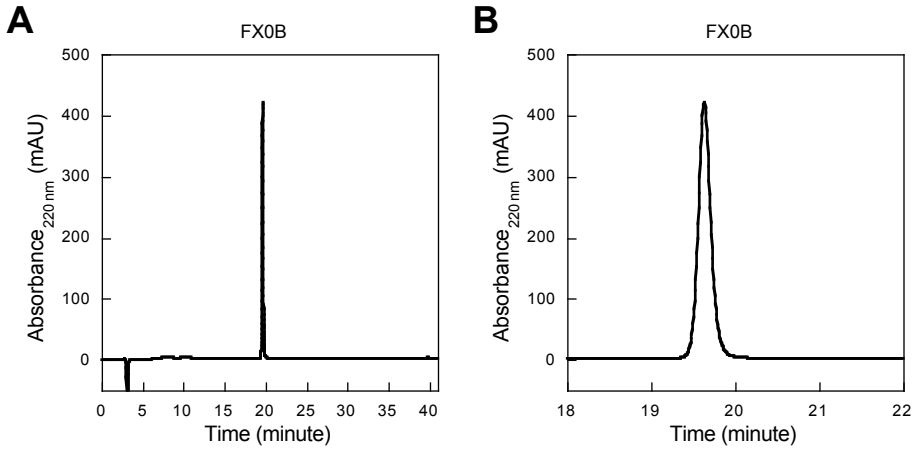

Fig. S14 The analytical HPLC chromatogram for purified FX0B peptide (A) and the zoomed in view of the desired peak (B).

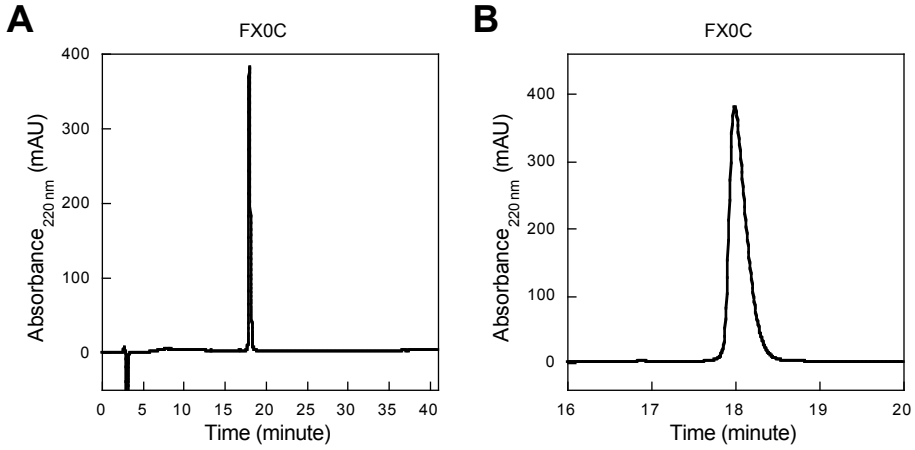

Fig. S15 The analytical HPLC chromatogram for purified FX0C peptide (A) and the zoomed in view of the desired peak (B).

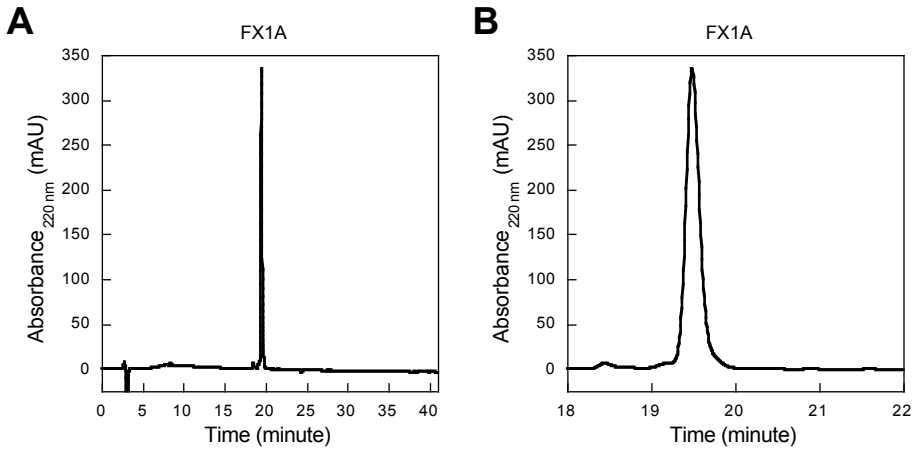

Fig. S16 The analytical HPLC chromatogram of purified FX1A peptide (A) and the zoomed in view of the desired peak (B).

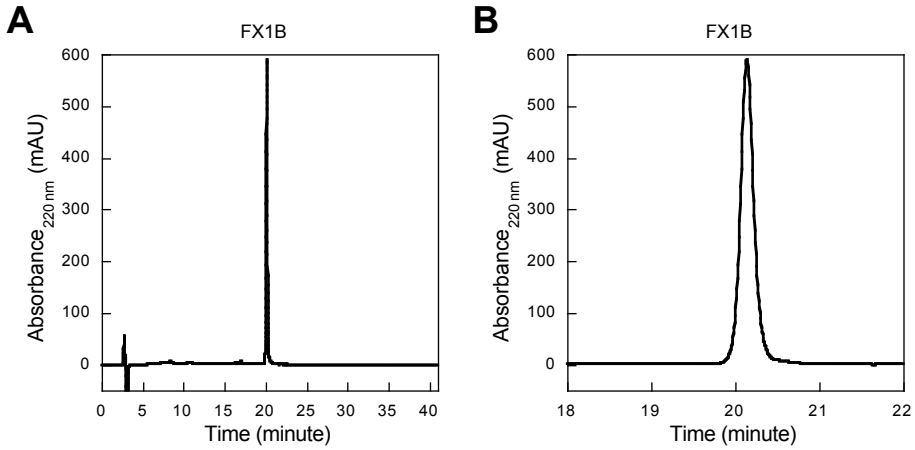

Fig. S17 The analytical HPLC chromatogram for purified FX1B peptide (A) and the zoomed in view of the desired peak (B).

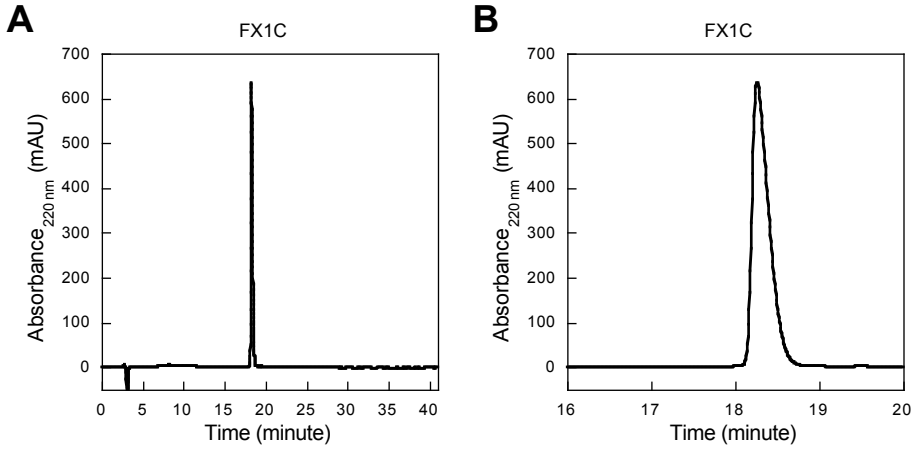

Fig. S18 The analytical HPLC chromatogram for purified FX1C peptide (A) and the zoomed in view of the desired peak (B).

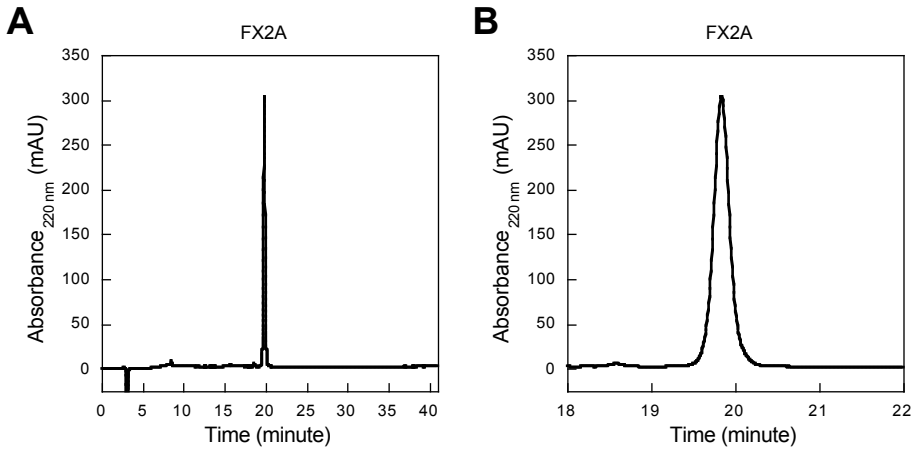

Fig. S19 The analytical HPLC chromatogram of purified FX2A peptide (A) and the zoomed in view of the desired peak (B).

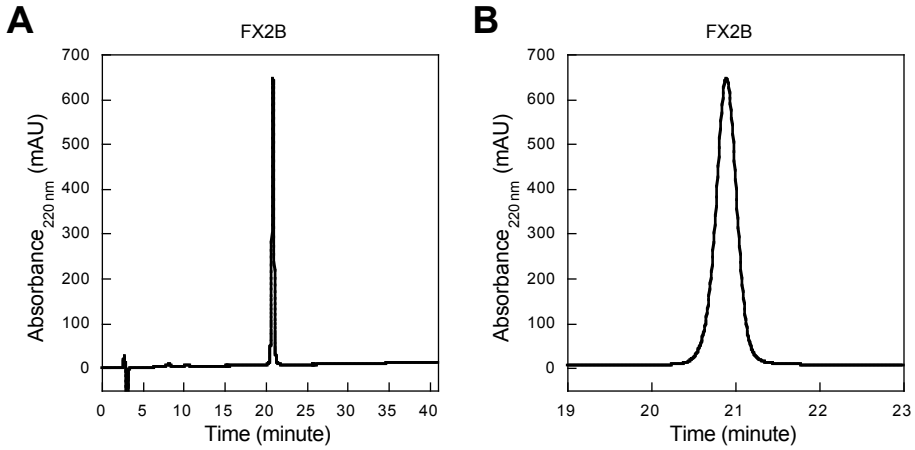

Fig. S20 The analytical HPLC chromatogram for purified FX2B peptide (A) and the zoomed in view of the desired peak (B).

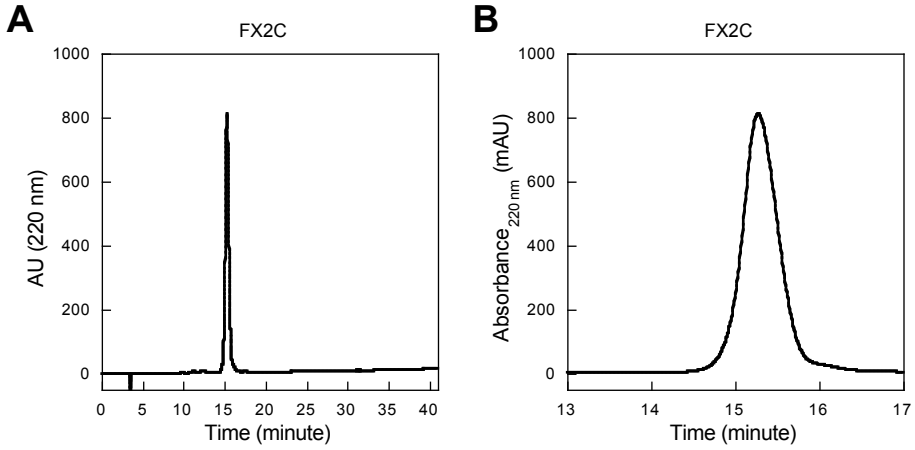

Fig. S21 The analytical HPLC chromatogram for purified FX2C peptide (A) and the zoomed in view of the desired peak (B).

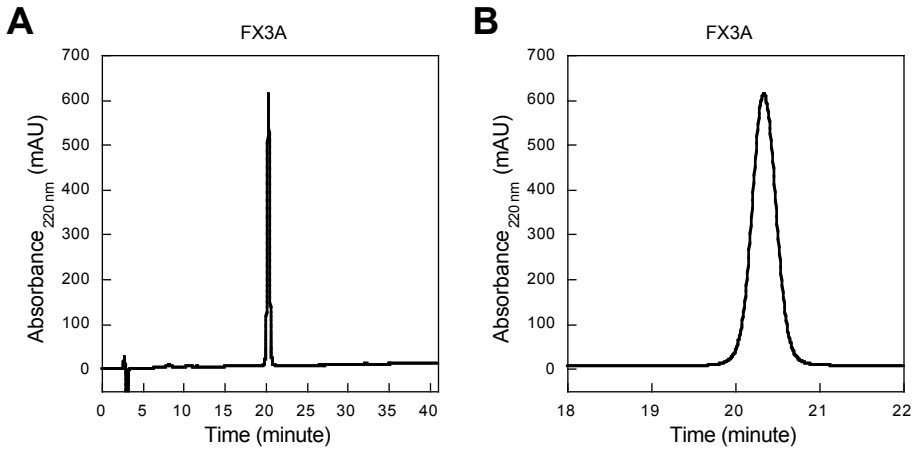

Fig. S22 The analytical HPLC chromatogram of purified FX3A peptide (A) and the zoomed in view of the desired peak (B).

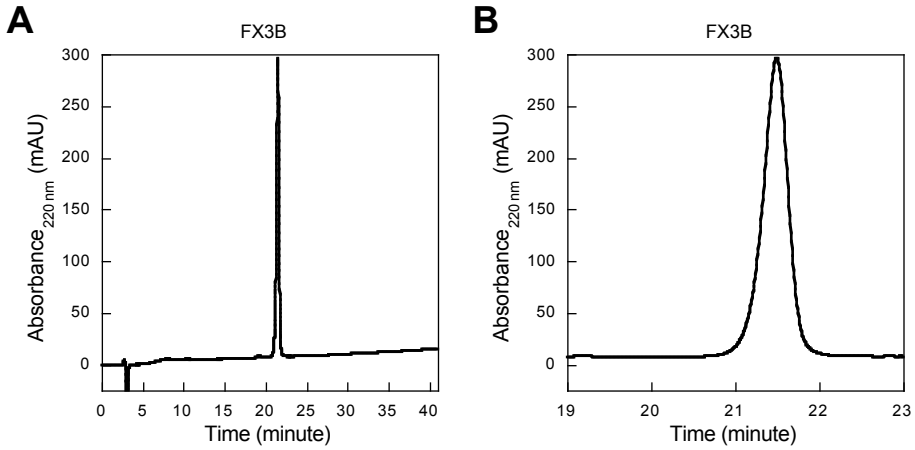

Fig. S23 The analytical HPLC chromatogram for purified FX3B peptide (A) and the zoomed in view of the desired peak (B).

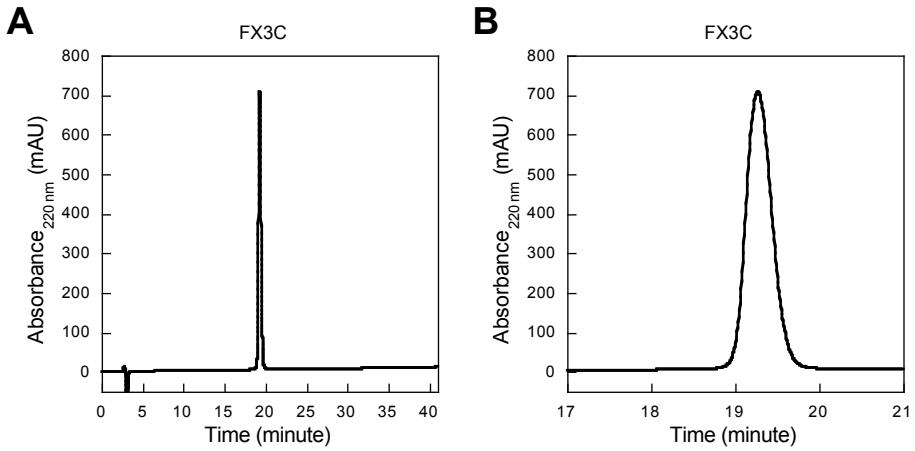

Fig. S24 The analytical HPLC chromatogram for purified FX3C peptide (A) and the zoomed in view of the desired peak (B).

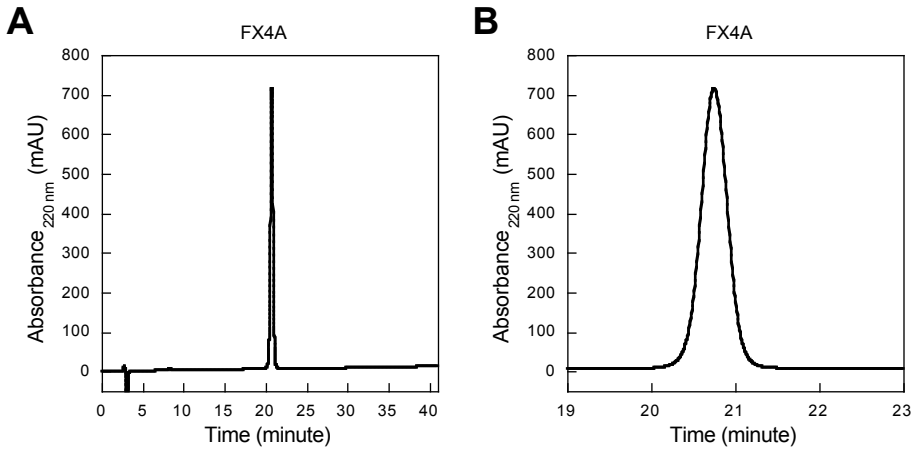

Fig. S25 The analytical HPLC chromatogram of purified FX4A peptide (A) and the zoomed in view of the desired peak (B).

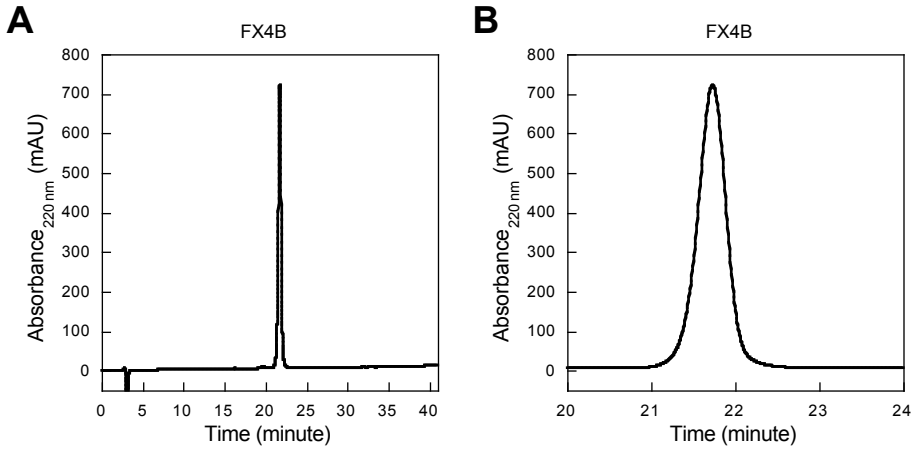

Fig. S26 The analytical HPLC chromatogram for purified FX4B peptide (A) and the zoomed in view of the desired peak (B).

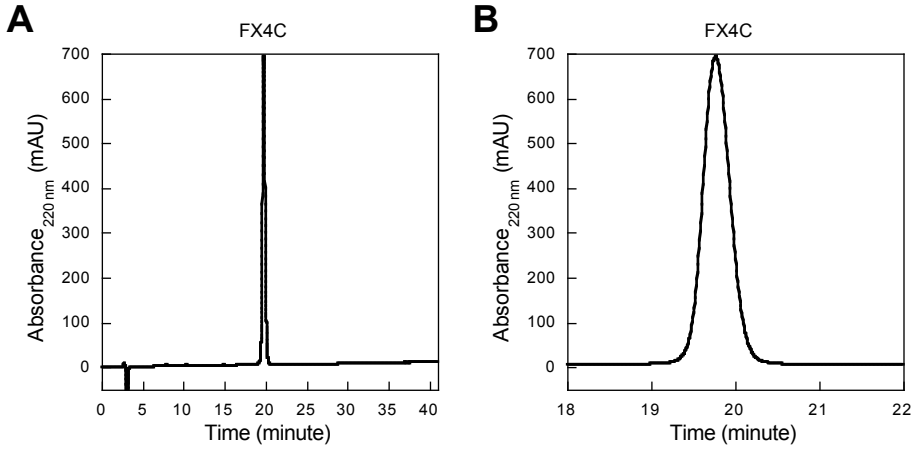

Fig. S27 The analytical HPLC chromatogram for purified FX4C peptide (A) and the zoomed in view of the desired peak (B).

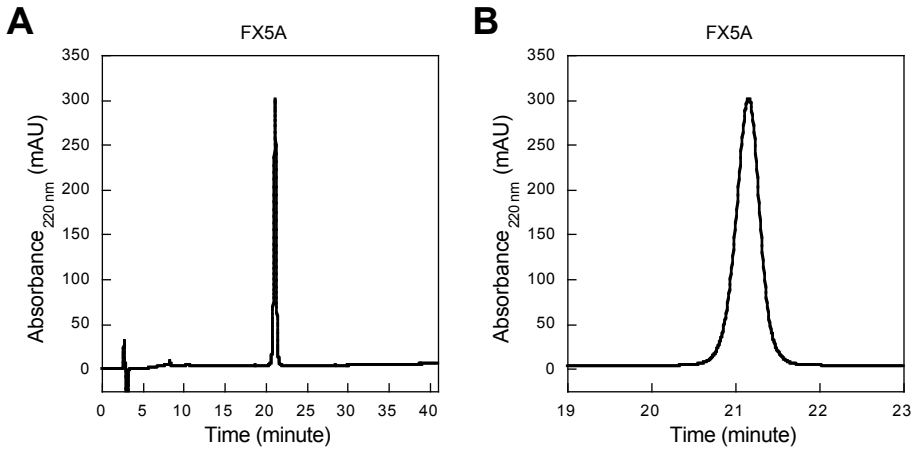

Fig. S28 The analytical HPLC chromatogram of purified FX5A peptide (A) and the zoomed in view of the desired peak (B).

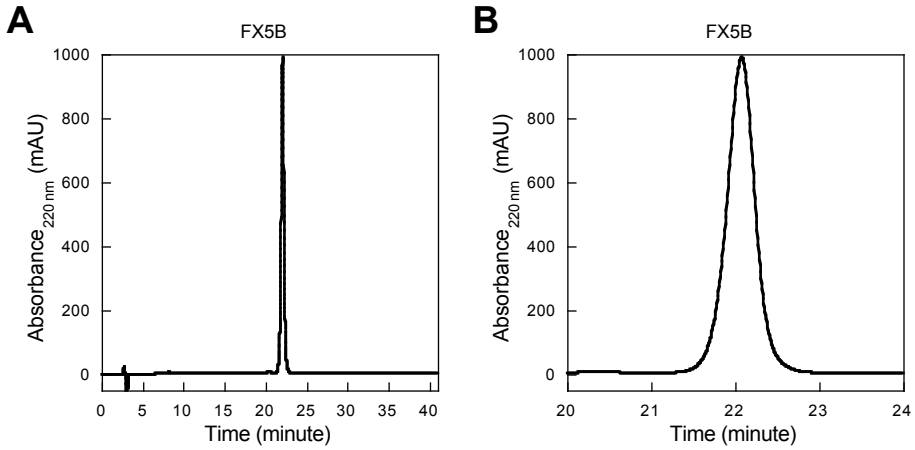

Fig. S29 The analytical HPLC chromatogram for purified FX5B peptide (A) and the zoomed in view of the desired peak (B).

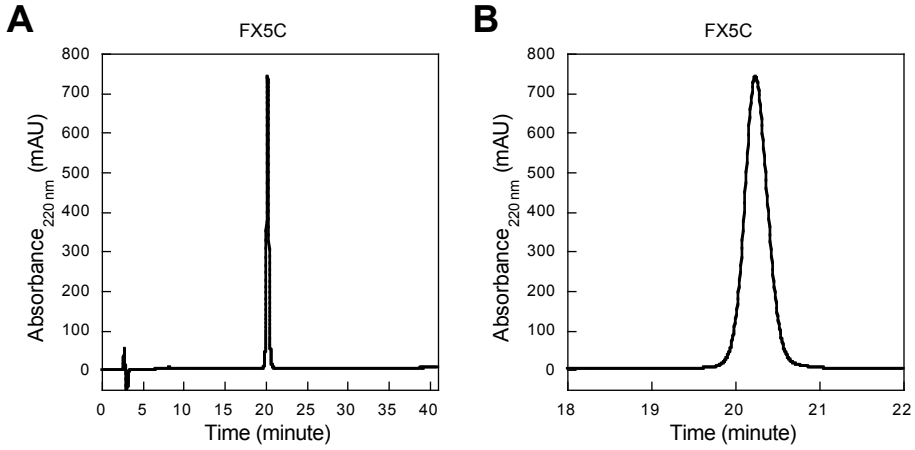

Fig. S30 The analytical HPLC chromatogram for purified FX5C peptide (A) and the zoomed in view of the desired peak (B).

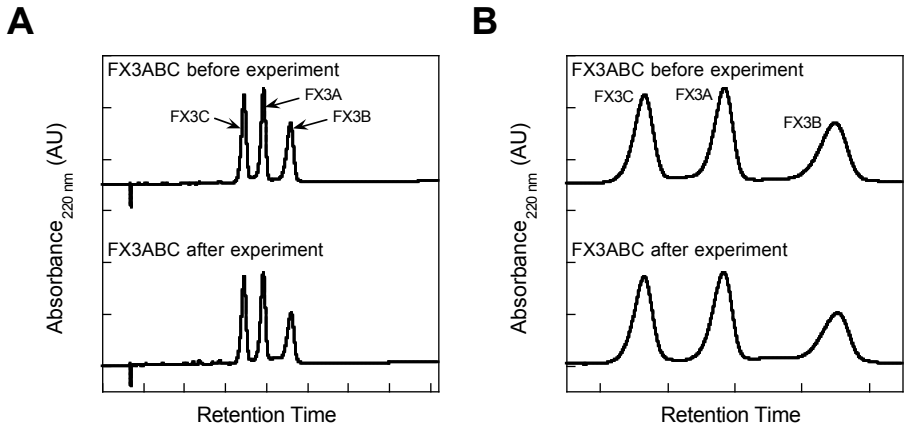

Fig. S31 The analytical HPLC chromatograms for combination FX3ABC before and after thermal folding/unfolding experiments (A) and the zoomed in view of the major peaks (B).

Table S1. Protocols for Preheating, Reversible Folding, and Reversible Unfolding for Combinations with T_m Approximately 50 °C (FX4ABC, FX5ABC)

Experiment	Temperature (°C)	Equilibration time
Preheating	5~75 (5 °C increments)	15 min
Reversible Cooling/ Folding	80	30 min
	70	30 min
	60	600 min
	50	1200 min
	40	1200 min
	30	1200 min
	20	1200 min
	10	1200 min
	2	1200 min
Reversible Heating/ Unfolding	4~18 (2 °C increments)	600 min
	20~38 (2 °C increments)	1200 min
	40~64 (2 °C increments)	2000 min
	66~70 (2 °C increments)	360 min
	72~84 (2 °C increments)	60 min

Table S2. Protocols for Preheating, Reversible Folding, and Reversible Unfolding for Combinations with T_m Approximately 40 °C (FX2ABC, FX3ABC, FX5BCC, FX5BBC, FX5AAB)

Experiment	Temperature (°C)	Equilibration time
Preheating	5~65 (5 °C increment)	15 min
Reversible Cooling/ Folding	70	30 min
	60	30 min
	50	600 min
	40	1200 min
	30	1200 min
	20	1200 min
	10	1200 min
	2	1200 min
Reversible Heating/ Unfolding	4	600 min
	6~24 (2 °C increment)	1200 min
	26~54 (2 °C increment)	2000 min
	56~60 (2 °C increment)	360 min
	62~74 (2 °C increment)	60 min

Table S3. Protocols for Preheating, Reversible Folding, and Reversible Unfolding for Combinations with T_m Approximately 30 °C (FX0ABC, FX1ABC, FX4BCC, FX4BBC, FX4AAB)

Experiment	Temperature (°C)	Equilibration time
Preheating	5~55 (5 °C increment)	15 min
Reversible Cooling/ Folding	60	30 min
	50	30 min
	40	600 min
	30	1200 min
	20	1200 min
	10	1200 min
	2	1200 min
Reversible Heating/ Unfolding	4~16 (2 °C increment)	1200 min
	18~44 (2 °C increment)	2000 min
	46~50 (2 °C increment)	360 min
	52~64 (2 °C increment)	60 min

Table S4. Protocols for Preheating, Reversible Folding, and Reversible Unfolding for Combinations with T_m Approximately 20 °C (FX3BCC, FX3BBC, FX3AAB)

Experiment	Temperature (°C)	Equilibration time
Preheating	5~45 (5 °C increment)	15 min
Reversible Cooling/ Folding	50	30 min
	40	30 min
	30	600 min
	20	1200 min
	10	1200 min
	2	1200 min
Reversible Heating/ Unfolding	4~8 (2 °C increment)	1200 min
	10~30 (2 °C increment)	2000 min
	32~40 (2 °C increment)	360 min
	42~54 (2 °C increment)	60 min

Table S5. Apparent Molecular Weight for FX*n*ABC Combinations as Determined by Sedimentation Velocity Experiments

Combination	MW (Calculated)	Major Species			Minor Species		
		MW	Sv	%	MW	Sv	%
FX0ABC	9517	8965	0.760	74.25	3173	0.380	25.28
FX1ABC	10319	10103	0.794	86.64	3045	0.357	12.80
FX2ABC	11120	11071	0.821	90.42	3322	0.368	8.73
FX3ABC	11925	11370	0.858	88.05	3134	0.363	11.83
FX4ABC	12726	11817	0.868	91.49	2230	0.286	8.07
FX5ABC	13527	12891	0.887	92.09	2130	0.267	7.14

Table S6. Thermodynamic Unfolding Parameters for Heterotrimeric Collagen Triple Helices

Combination	T_m (°C)	ΔH_{Tm} (kcal/mol)	ΔS_{Tm} (kcal/mol ·K)	$\Delta G_{44°C}$ (kcal/mol)
FX0ABC	28.68 ± 0.07	140 ± 4	0.43 ± 0.01	3.79 ± 0.10[a]
FX1ABC	36.91 ± 0.09	156 ± 5	0.47 ± 0.01	7.35 ± 0.24[a]
FX2ABC	41.00 ± 0.10	115 ± 3	0.33 ± 0.01	9.79 ± 0.16
FX3ABC	47.50 ± 0.08	126 ± 3	0.36 ± 0.01	12.54 ± 0.05
FX4ABC	51.37 ± 0.12	117 ± 4	0.32 ± 0.01	13.87 ± 0.06
FX5ABC	53.07 ± 0.13	113 ± 3	0.31 ± 0.01	15.91 ± 0.06
FX3BCC	29.25 ± 0.07	99 ± 2	0.29 ± 0.01	6.09 ± 0.12[a]
FX4BCC	39.51 ± 0.13	102 ± 3	0.29 ± 0.01	9.38 ± 0.41
FX5BCC	45.69 ± 0.14	84 ± 2	0.23 ± 0.01	11.65 ± 0.09
FX3BBC	26.18 ± 0.18	65 ± 2	0.18 ± 0.01	7.04 ± 0.23[a]
FX4BBC	33.90 ± 0.22	60 ± 2	0.16 ± 0.01	8.56 ± 0.66
FX5BBC	42.57 ± 0.20	66 ± 2	0.17 ± 0.01	10.92 ± 0.18
FX3AAB	23.89 ± 0.06	151 ± 3	0.47 ± 0.01	0.70 ± 0.10[a]
FX4AAB	32.12 ± 0.07	147 ± 3	0.45 ± 0.01	5.20 ± 0.13[a]
FX5AAB	37.77 ± 0.10	111 ± 3	0.32 ± 0.01	8.01 ± 0.87

[a] The values were extrapolated to 44 °C based on ΔH_{Tm} and T_m.

Experimental procedures

General section

All of the chemical reagents except those indicated otherwise were purchased from Sigma-Aldrich. *N*,*N*-Diisopropylethylamine (DIEA), acetic anhydride (Ac_2O), triisopropylsilane (TIS), α-cyano-4-hydroxycinnamic acid were purchased from Acros Piperidine and trifluoroacetic acid (TFA) were purchased from Alfa Aesar. Hexanes was purchased from Duksan. Dimethylformamide (DMF), acetonitrile, and methanol were from Merck. NovaSyn® TGR resin, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU), and *N*-9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Novabiochem. All reagents and solvents were used without further purification. Sep-Pak® Plus Short tC18 cartridges were purchased from Waters.

Analytical reverse phase (RP)-HPLC was performed on an Agilent 1200 series chromatography system using a Vydac C_{18} column (4.6 mm diameter, 250 mm length). Preparative RP-HPLC was performed on a Waters Breeze chromatography system using a C_{18} column (22 mm diameter, 250 mm length). Mass spectrometry of the peptides was performed on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometer (Bruker Autoflex Speed) using α-cyano-4-hydroxycinnamic acid as the matrix. Peptide concentration was determined using a UV-vis spectrometer (Jasco V-650). Thermal denaturation was monitored by circular dichroism spectroscopy using a circular dichroism spectrometer (Jasco J-815).

Peptide synthesis

Peptides were synthesized by solid phase peptide synthesis using Fmoc (N^{α}-fluorenylmethyloxycarbonyl)-based chemistry. For a typical peptide synthesis, NovaSyn®TGR resin (0.05 mmol) was swollen in *N, N*-dimethylformamide (DMF, 5 mL) for 30 minutes. A mixture of appropriately protected Fmoc amino acid (3 equivalents) and HATU (3 equivalents) was dissolved in DMF (1 mL). Diisopropylethylamine (DIEA, 5 equivalents) was then added to the solution and mixed thoroughly. The solution was then applied to the resin. The vial that contained the solution was rinsed with DMF (2x1 mL) and added to the reaction. Protected dipeptide Fmoc-Asp(tBuO)-(Dmb)Gly-OH was used to incorporate every Asp-Gly motif in the sequence. All residues were coupled for 10 minutes at room temperature with shaking. After each coupling, the resin was washed with DMF (5 mL, 5x1 min). The Fmoc on the resin-bound protected peptide was then removed using 20% piperidine/DMF (5 mL, 3x8 min) and then washed with DMF (5 mL, 5x1 min). For capping with acetic anhydride (Ac_2O), a solution of Ac_2O (20 equivalents), DIEA (20 equivalents), DMF (1 mL) was added to the resin. The reaction was shaken for 2 hours. The resin was

subsequently washed with DMF (5 mL, 5x1 min) and methanol (5 mL), and was lyophilized overnight.

Peptides were deprotected and cleaved off the resin by treating the resin with 5 mL 95:5 trifluoroacetic acid (TFA)/triisopropylsilane and shaken for 2 hours. The solution was then filtered through glass wool and the resin was washed with TFA (3x1 mL). The combined filtrate was evaporated by a gentle stream of air. The resulting material was washed with hexanes (3x3 mL), dissolved in water, and lyophilized. The peptide (1 mg mL^{-1} aqueous solution) was analyzed using analytical RP-HPLC on a 25 cm C_{18} column (dia 4.6 mm) with flow rate 1 $mL \cdot min^{-1}$, temperature 25°C, linear 1 $\% \cdot min^{-1}$ gradient from 100% A to 0% A (solvent A: 99.9% water,0.1% TFA; solvent B: 90% acetonitrile, 10% water, 0.1% TFA). Peptides were purified to higher than 90% purity by Sep-Pak® Plus Short tC18 cartridges using an appropriate percentage of B solvent and by reverse phase HPLC using a preparative C_{18} column with flow rate 10 $mL \cdot min^{-1}$, temperature 25°C, linear 0.5 $\% \cdot min^{-1}$ gradient. Appropriate linear gradients of solvent A and solvent B were used for each peptide to place the retention time for the desired peptide between 20 and 30 minutes. These gradients are listed individually for each peptide (vide infra); for example, PLG06_16 was used to purify FX0A, representing the linear gradient from 6 % B to 16 % B (flow rate 10 $mL \cdot min^{-1}$, temperature 25°C, linear 0.5 $\% \cdot min^{-1}$ gradient). The identity of the peptides were confirmed by MALDI-TOF.

FX0A

(Ac-Tyr-Gly-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Lys-Gly-Pro-Lys-Gly-NH_2)

The peptide was synthesized using 203.7 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 387.0 mg of resin (89.1% yield). Cleavage gave 135.9 mg of crude peptide (77.8% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 18.9 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG06_16) to 93.3% purity (25.1 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{137}H_{212}N_{40}O_{47}$ [MH^+]: 3170.550; observed: 3170.556.

FX0B

(Ac-Tyr-Gly-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Asp-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-NH_2)

The peptide was synthesized using 203.4 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 386.2 mg of resin (93.9% yield). Cleavage gave 136.0 mg of crude peptide (88.3% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 19.6 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B

solvent) and reverse-phase HPLC with a preparative C18 column (PLG08_18) to 91.5% purity (23.1 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{127}H_{177}N_{35}O_{58}$ [MH^+]: 3121.205; observed: 3121.197.

FX0C

(Ac-Tyr-Gly-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-NH_2)

The peptide was synthesized using 200.3 mg (0.050 mmol) of NovaSyn®TGR resin. Synthesis gave 385.7 mg of resin (86.1% yield). Cleavage gave 134.4 mg of crude peptide (75.6% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 18.0 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG06_16) to 93.5% purity (58.2 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{141}H_{226}N_{42}O_{45}$ [MH^+]: 3228.676; observed: 3228.701.

FX1A

(Ac-Tyr-Gly-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Pro-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Lys-Gly-Pro-Lys-Gly-NH_2)

The peptide was synthesized using 205.0 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 436.5 mg of resin (>99% yield). Cleavage gave 149.5 mg of crude peptide (67.5% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 19.5 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG07_17) to 90.9% purity (12.5 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{149}H_{229}N_{43}O_{51}$ [MH^+]: 3437.672; observed: 3437.682.

FX1B

(Ac-Tyr-Gly-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Asp-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-NH_2)

The peptide was synthesized using 231.4 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 426.8 mg of resin (92.4% yield). Cleavage gave 145.7 mg of crude peptide (89.0% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 20.2 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG09_19) to 94.0% purity (14.0 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{139}H_{194}N_{38}O_{62}$ [MH^+]: 3388.327; observed: 3388.493.

FX1C

(Ac-Tyr-Gly-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-NH_2)

The peptide was synthesized using 205.4 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 441.5 mg of resin (>99% yield). Cleavage gave 215.8 mg of crude peptide (94.9% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 18.3 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG07_17) to 92.0% purity (8.7 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{153}H_{243}N_{45}O_{49}$ [MH^+]: 3495.798; observed: 3495.739.

FX2A

(Ac-Tyr-Gly-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Lys-Gly-Pro-Lys-Gly-NH_2)

The peptide was synthesized using 227.8 mg (0.057 mmol) of NovaSyn®TGR resin. Synthesis gave 436.3 mg of resin (>99% yield). Cleavage gave 229.1 mg of crude peptide (>99% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 19.9 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG08_18) to 91.4% purity (35.3 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{161}H_{246}N_{46}O_{55}$ [MH^+]: 3704.794; observed: 3704.716.

FX2B

(Ac-Tyr-Gly-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Asp-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-NH_2)

The peptide was synthesized using 232.5 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 434.6 mg of resin (88.2% yield). Cleavage gave 146.1 mg of crude peptide (86.3% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 20.9 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG10_20) to 92.4% purity (54.4 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{151}H_{211}N_{41}O_{66}$ [MH^+]: 3655.449; observed: 3655.577.

FX2C

(Ac-Tyr-Gly-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-NH_2)

The peptide was synthesized using 201.7 mg (0.050 mmol) of NovaSyn®TGR resin. Synthesis gave 377.7 mg of resin (>99% yield). Cleavage gave 118.5 mg of crude peptide (86.3% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 15.3 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG07_17) to 93.6% purity (29.8 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{165}H_{260}N_{48}O_{53}$ [MH^+]: 3762.920; observed: 3762.901.

FX3A

(Ac-Tyr-Gly-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Lys-Gly-Pro-Lys-Gly-NH_2)

The peptide was synthesized using 230.1 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 403.9 mg of resin (68.5% yield). Cleavage gave 149.3 mg of crude peptide (93.0% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 20.3 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG09_19) to 93.9% purity (54.0 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{173}H_{263}N_{49}O_{59}$ [MH^+]: 3971.916; observed: 3972.023.

FX3B

(Ac-Tyr-Gly-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Asp-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-NH_2)

The peptide was synthesized using 227.9 mg (0.050 mmol) of NovaSyn®TGR resin. Synthesis gave 432.1 mg of resin (84.8% yield). Cleavage gave 143.6 mg of crude peptide (84.1% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 21.5 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG10_20) to 92.0% purity (59.3 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{163}H_{228}N_{44}O_{70}$ [MH^+]: 3922.571; observed: 3922.623.

FX3C

(Ac-Tyr-Gly-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-NH_2)

The peptide was synthesized using 228.3 mg (0.050 mmol) of NovaSyn®TGR resin. Synthesis gave 404.1 mg of resin (66.3% yield). Cleavage gave 159.2 mg of crude peptide (97.2% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 19.3 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG08_18) to 95.3% purity (48.4 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{177}H_{277}N_{51}O_{57}$ [MH^+]: 4030.042; observed: 4030.157.

FX4A

(Ac-Tyr-Gly-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Lys-Gly-Pro-Lys-Gly-NH_2)

The peptide was synthesized using 228.5 mg (0.050 mmol) of NovaSyn®TGR resin. Synthesis gave 409.1 mg of resin (67.3% yield). Cleavage gave 177.0 mg of crude peptide (>99% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 20.7 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG09_19) to 94.0% purity (53.8 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{197}H_{297}N_{55}O_{67}$ [MH^+]: 4239.038; observed: 4239.284.

FX4B

(Ac-Tyr-Gly-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Asp-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-NH_2)

The peptide was synthesized using 231.1 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 430.3 mg of resin (76.4% yield). Cleavage gave 144.4 mg of crude peptide (86.6% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 21.7 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG11_21) to 92.3% purity (48.0 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{175}H_{245}N_{47}O_{74}$ [MH^+]: 4189.693; observed: 4190.173.

FX4C

(Ac-Tyr-Gly-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-NH_2)

The peptide was synthesized using 230.9 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 414.9 mg of resin (64.7% yield). Cleavage gave 156.7 mg of crude peptide (92.0% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 19.8 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG08_18) to 94.5% purity (47.0 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{189}H_{294}N_{54}O_{61}$ [MH^+]: 4297.164; observed: 4297.260.

FX5A

(Ac-Tyr-Gly-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Lys-Gly-Pro-Lys-Gly-NH_2)

The peptide was synthesized using 204.7 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 412.1 mg of resin (71.6% yield). Cleavage gave 169.7 mg of crude peptide (89.3% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 21.2 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG08_18) to 93.0% purity (57.4 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{197}H_{297}N_{55}O_{67}$ [MH^+]: 4506.160; observed: 4506.198.

FX5B

(Ac-Tyr-Gly-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Asp-Hyp-Gly-Pro-Asp-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Asp-Hyp-Gly-NH_2)

The peptide was synthesized using 203.8 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 414.5 mg of resin (75.9% yield). Cleavage gave 153.3 mg of crude peptide (86.8% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 22.1 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG11_21) to 92.9% purity (29.3 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{187}H_{262}N_{50}O_{78}$ [MH^+]: 4456.815; observed: 4557.172.

FX5C

(Ac-Tyr-Gly-Gly-Lys-Hyp-Gly-Pro-Asp-Gly-Pro-Asp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Lys-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-Lys-Hyp-Gly-NH_2)

The peptide was synthesized using 204.6 mg (0.051 mmol) of NovaSyn®TGR resin. Synthesis gave 406.9 mg of resin (66.8% yield). Cleavage gave 177.4 mg of crude peptide (95.0% yield). Retention time on the reverse-phase HPLC with an analytical C18 column was 20.2 minutes. Crude peptide was purified using a Sep-Pak® Plus Short tC_{18} cartridge (25% B solvent) and reverse-phase HPLC with a preparative C18 column (PLG08_18) to 94.3% purity (31.2 mg). Identity of the peptide was confirmed by MALDI-TOF mass spectrometry. Calculated for $C_{201}H_{311}N_{57}O_{65}$ [MH^+]: 4564.286; observed: 4564.697.

UV-vis spectroscopy

Peptide stock concentration was determined on a UV-Vis spectrophotometer (Jasco V-650). Purified peptide was used to prepare 10 mM stock solution. UV-vis spectroscopy was utilized to determine the concentration of peptides as described by Edelhoch.[1, 2] After each addition of the peptide stock, the solution was equilibrated for 15 minutes, and the absorption of the phenol group on Tyr at 276 (ε_{276} = 1455), 278 (ε_{278} = 1395), 280 (ε_{280} = 1285), 282 (ε_{282} = 1220) nm[1, 2] was measured at 25°C. The absorbance at these wavelengths was baseline corrected by the absorbance at 335 or 400 nm. The stock concentration was determined by linear regression based on the Beer-Lambert law. Stock concentrations were determined from experiments giving linear regression R values equal to or higher than 0.999.

Circular dichroism spectroscopy

Circular dichroism (CD) data were acquired on a Jasco J-815 spectrometer using 1 mm pathlength quartz cells sealed with DuraSeal®. Samples were prepared to a total peptide concentration of 200 μM in 10 mM sodium phosphate buffer (pH 7.0). Heating and cooling of the samples were monitored by CD at 225 nm. For each temperature, 61 data points were collected in 1 minute and were averaged.

For preliminary assessment of the different combinations, the samples were prepared by mixing the constituting peptides in different ratios. For a given number of inserted Pro-Hyp-Gly triplets (*n*), 10 different combinations (AAA, BBB, CCC, AAB, ABB, AAC, ACC, BBC, BCC, ABC) were studied. For example, to prepare the combination FX3ABC, 4 μL 6.87 mM FX3A, 4 μL 6.87 mM FX3B, and 4 μL 6.87 mM FX3C were mixed in 400 μL 10 mM sodium phosphate buffer (pH 7.0). A total of 60 different combinations were assessed. The different combinations were heated from 5°C to 95°C in 5 degree increments. The samples were then cooled to 5°C in 10-degree decrements. Upon reaching the target temperature, the samples were equilibrated for 15 minutes prior to recording the CD signal at

225 nm.

For reversible thermal folding and unfolding experiments, the samples were preheated from 5°C to a temperature necessary to form fully unfolded monomers for the combination of interest in 5 degree increments (Tables S1~S4), equilibrating for 15 minutes at each temperature. Preheating dissociates misaligned triple helices and homotrimers of individual peptides, and allows for formation of fully unfolded monomers.[3] The samples were then cooled to 5°C in 10-degree decrements and then to 2°C, with equilibration times as long as 1200 minutes (Tables S1~S4). The samples were then heated from 2°C in 2-degree increments until an unfolded baseline was formed (Tables S1~S4), with equilibration times as long as 2000 minutes. Samples with similar melting temperatures were measured using the same equilibration protocols. Superposition of the cooling and heating cycles was indicative of reversible thermal unfolding and folding with thermal equilibrium at each temperature.

The folded baseline was obtained by linear regression of the consecutive linear data points at temperatures lower than the transition temperature. The unfolded baseline was obtained by linear regression of the consecutive linear data points at temperatures higher than the melting transition. The fraction folded at a given temperature F(T) was derived from the CD signal and the corresponding folded and unfolded baselines (equation 1); $\theta(T)$ is the observed CD signal at temperature T; $\theta_T(T)$ and $\theta_M(T)$ are the estimated CD signals for the folded trimer and unfolded monomer at temperature T based on the folded baseline and unfolded baseline, respectively.[4-6]

$$F(T) = \frac{\theta(T)-\theta_M(T)}{\theta_T(T)-\theta_M(T)} \qquad \text{equation (1)}$$

The reversible thermal transition between the folded and unfolded states was assumed to be a two state equilibrium between folded trimers (T) and unfolded monomers (M) (equation 2).[4]

$$T \rightleftharpoons 3M \qquad \text{equation (2)}$$

The equilibrium constant K(T) at temperature T was derived from equation 3, in which c_0 is a total peptide concentration,[4-6] c_T and c_M are the concentration of folded trimer and unfolded monomer at temperature T, respectively.

$$K(T) = \frac{c_M^3}{c_T} = \frac{3c_0^2[1-F(T)]^3}{F(T)} \qquad \text{equation (3)}$$

At the midpoint transition temperature (T_m), the fraction folded $F(T_m)$ would be 0.5. Accordingly, the equilibrium constant at the midpoint transition temperature would be expressed as in equation 4.

$$K(T_m) = \frac{3c_0^3\left(1-0.5\right)^3}{0.5c_0} = \frac{3}{4}c_0^2 \qquad \text{equation (4)}$$

According to thermodynamics, the relationship between Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) is defined as equation 5.

$$\Delta G(T) = - RT\ln K(T) = \Delta H - T\Delta S \qquad \text{equation (5)}$$

As such, ΔS_{Tm} is related to T_m, ΔH_{Tm}, and c_0 by equation 6.[4-6]

$$\Delta S_{T_m} = \frac{\Delta H_{T_m}}{T_m} + R\ln\left(\frac{3c_0^2}{4}\right) \qquad \text{equation (6)}$$

Assuming ΔH and ΔS are independent of temperature, meaning $\Delta H = \Delta H_{Tm}$ and $\Delta S = \Delta S_{Tm}$. The unfolding free energy at temperature $\Delta G_{unfold}(T)$ is further expressed as equation 7, and the equilibrium constant K(T) is further expressed as equation 8.[4-6]

$$\Delta G_{unfold}(T) = \left[\Delta H_{T_m}\left(1-\frac{T}{T_m}\right) - RT\ln\left(\frac{3c_0^2}{4}\right)\right] \qquad \text{equation (7)}$$

$$K(T) = \exp\left[\frac{\Delta H_{T_m}}{RT}\left(\frac{T}{T_m}-1\right) + \ln\left(\frac{3c_0^2}{4}\right)\right] \qquad \text{equation (8)}$$

The fraction folded F(T) was expressed as a function of temperature in equation 9. The temperature dependent fraction folded data was fit to equation 9 to give ΔH_{Tm} and T_m (Tables 1 and S6). Data from at least two folding experiments and two unfolding experiments were included in every fit. ΔS_{Tm} was derived from ΔH_{Tm} and T_m using equation 6 (Table S6). ΔG_{unfold} at 44°C was directly derived from the equilibrium constant using the fraction folded data at 44°C (equation 5). If insufficient fraction folded and fraction unfolded was present at 44°C, ΔG_{unfold} at 44°C was extrapolated from ΔH_{Tm} and T_m using equation 7.

$$F(T) = 1 + \left\{\frac{\sqrt{27 + \exp\left[\frac{\Delta H}{RT}\left(\frac{T}{T_m}-1\right)\right]}\cdot\exp\left[\frac{\Delta H}{RT}\left(\frac{T}{T_m}-1\right)\right]}{8\times 3^{\frac{3}{2}}} - \frac{1}{8}\exp\left[\frac{\Delta H}{RT}\left(\frac{T}{T_m}-1\right)\right]\right\}^{\frac{1}{3}} - \frac{\exp\left[\frac{\Delta H}{RT}\left(\frac{T}{T_m}-1\right)\right]}{12\left\{\frac{\sqrt{27 + \exp\left[\frac{\Delta H}{RT}\left(\frac{T}{T_m}-1\right)\right]}\cdot\exp\left[\frac{\Delta H}{RT}\left(\frac{T}{T_m}-1\right)\right]}{8\times 3^{\frac{3}{2}}} - \frac{1}{8}\exp\left[\frac{\Delta H}{RT}\left(\frac{T}{T_m}-1\right)\right]\right\}^{\frac{1}{3}}}$$

Equation (9)

Analytical ultracentrifugation

Samples (450 µL) were prepared with a total peptide concentration of 180 µM in 10 mM sodium phosphate (pH 7.0) using the appropriate peptides to form the intended combinations (vide supra). The samples were preheated from 5°C to a temperature necessary to form fully unfolded monomers for the combination of interest in 5 degree increments (Tables S1~S4), equilibrating for 15 minutes at each temperature. The samples were then cooled to 5°C in 10

degree decrements and then to 2°C, with equilibration times as long as 1200 minutes (Tables S1~S4). The samples were then kept at 4°C for the sedimentation velocity experiments.

Sedimentation velocity experiments were performed using a Beckman Coulter ProteomeLab XL-I analytical ultracentrifuge with an AN 60Ti rotor. Sedimentation velocity data of the various combinations were collected at 280 nm at 4°C with rotor speed of 60,000 rpm (250,000 x g). Solvent density, viscosity and protein's partial specific volumes were calculated using the software Sednterp1.[7] The sedimentation coefficients, molecular weights and population distributions were analyzed using the Sedfit2 program.[8]

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