Supporting Information

PEG-based Changes to β-sheet Protein Conformational and Proteolytic Stability Depend on Conjugation Strategy and Location

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1. Protein Synthesis, Purification, and Characterization

Proteins **16N**, **16Np**, **18N**, **18Np**, **19Q**, **19Qp**, **19X**, **19Xp**, **19Z**, **19Zp**, **19N**, **19Np**, **19Nbp**, **23N**, **23Np**, **27N**, **27Np**, **29N**, **29Np**, **32N**, and **32Np** (sequences shown in Table S1) were synthesized previously.[1, 2] The remaining WW variants shown in Table 1 were synthesized as C-terminal acids by Fmoc-based microwave-assisted solid-phase peptide synthesis as described previously,[2] using the following reagents: Fmoc-Gly-loaded Novasyn Wang resin (EMD Biosciences); standard Fmoc-protected α-amino acids with acid-labile side-chain protecting groups (EMD Biosciences or Advanced ChemTech); previously synthesized Fmoc-L-GlnPEG$_4$-OH [18-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-15-oxo-2,5,8,11-tetraoxa-14-azanonadecan-19-oic acid],[2] used to prepare proteins **16Qp**, **18Qp**, **23Qp**, **27Qp**, **29Qp**, and **32Qp**; commercially available Fmoc-L-4-azidohomoalanine, used to prepare proteins **16X**, **18X**, **23X**, **27X**, **29X**, and **32X**; previously synthesized PEG-alkyne 2,5,8,11-tetraoxatetradec-13-yne[3] used to prepare **16Xp**, **18Xp**, **23Xp**, **27Xp**, **29Xp**, and **32Xp** from proteins **16X**, **18X**, **23X**, **27X**, **29X**, and **32X** via the copper (I) catalyzed azide-alkyne cycloaddition[2]; previously synthesized Fmoc-L-PrF-OH N-[(9H-Fluoren-9-ylmethoxy)-O-2-propyn-1yl-L-tyrosine[4], used to prepare proteins **16Z**, **18Z**, **23Z**, **27Z**, **29Z**, and **32Z**; commercially available PEG-azide 13-azido-2,5,8,11-tetraoxatridecane, used to prepare proteins **16Zp**, **18Zp**, **23Zp**, **27Zp**, **29Zp**, and **32Zp** from proteins **18Z**, **23Z**, **27Z**, **29Z**, and **32Z** via the copper (I) catalyzed azide-alkyne cycloaddition[2]; previously synthesized Fmoc-L-(AsnPEG$_4$)$_2$-OH [(*S*)-17-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-15-oxo-14-(2,5,8,11-tetraoxatridecan-13-yl)-2,5,8,11-tetraoxa-14-azaoctadecan-18-oic acid],[2] used to prepare proteins **16Nbp**, **18Nbp**, **23Nbp**, **27Nbp**, **29Nbp**, and **32Nbp**; 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxybenzotriazole hydrate (HOBt) from Advanced ChemTech for amino acid activation; 20% piperidine in N,N-dimethylformamide for removal of the Fmoc protecting group from the N-terminal α-amine; a solution of a solution of phenol (0.0625 g), water (62.5 μL), thioanisole (62.5 μL), ethanedithiol (31 μL) and triisopropylsilane (12.5 μL) in trifluoroacetic acid (TFA, 1 mL) for cleaving the protein from resin and globally removing acid-labile side-chain protecting groups. Proteins were precipitated from the TFA solution by addition of diethyl ether (~40 mL). Following centrifugation, the ether was decanted, and the pellet was dissolved in ~40mL 1:1 H_2O/MeCN, then flash frozen over dry ice in acetone and lyophilized to remove volatile impurities. The resulting powder was stored at -20°C until purification.

Proteins were purified by preparative reverse-phase high performance liquid chromatography (HPLC) on a C18 column using a linear gradient of water in acetonitrile with 0.1% v/v TFA. Fractions containing the desired protein product were pooled, frozen, and lyophilized. Proteins were identified by electrospray ionization time of flight mass spectrometry (ESI-TOF); expected and observed exact masses mass spectra appear in Table S1 and spectra appear in Figures S1–S41. Protein purity was assessed by Analytical HPLC (traces are shown in Figures S42–S86).

Table S1. Sequences for WW variants, along with expected and observed exact masses from ESI-TOF MS experiments.

Peptide	Sequence	z	Expected $[M+z\cdot H^+]/z$	Observed $[M+ z\cdot H^+]/z$
16Q	H_2N-KLPPGWEKRM**Q**RSSGRVYYFNHITNASQFERPSG-COOH	4	1006.76	1006.74
16Qp	H_2N-KLPPGWEKRM**Q**RSSGRVYYFNHITNASQFERPSG-COOH	4	1054.29	1054.29
16X	H_2N-KLPPGWEKRM**X**RSSGRVYYFNHITNASQFERPSG-COOH	3	1341.51	1341.34
16Xp	H_2N-KLPPGWEKRM**X**RSSGRVYYFNHITNASQFERPSG-COOH	3	1408.72	1408.71
16Z	H_2N-KLPPGWEKRM**Z**RSSGRVYYFNHITNASQFERPSG-COOH	4	1025.02	1025.03
16Zp	H_2N-KLPPGWEKRM**Z**RSSGRVYYFNHITNASQFERPSG-COOH	4	1083.30	1083.30
16Nbp	H_2N-KLPPGWEKRM**N**RSSGRVYYFNHITNASQFERPSG-COOH	4	1098.32	1098.31
18Q	H_2N-KLPPGWEKRMSR**Q**SGRVYYFNHITNASQFERPSG-COOH	4	1006.76	1006.75
18Qp	H_2N-KLPPGWEKRMSR**Q**SGRVYYFNHITNASQFERPSG-COOH	4	1054.29	1054.29
18X	H_2N-KLPPGWEKRMSR**X**SGRVYYFNHITNASQFERPSG-COOH	3	1341.34	1341.33
18Xp	H_2N-KLPPGWEKRMSR**X**SGRVYYFNHITNASQFERPSG-COOH	3	1408.72	1408.70
18Z	H_2N-KLPPGWEKRMSR**Z**SGRVYYFNHITNASQFERPSG-COOH	4	1025.02	1025.00
18Zp	H_2N-KLPPGWEKRMSR**Z**SGRVYYFNHITNASQFERPSG-COOH	4	1083.30	1083.30
18Nbp	H_2N-KLPPGWEKRMSR**N**SGRVYYFNHITNASQFERPSG-COOH	4	1098.32	1098.31
23Q	H_2N-KLPPGWEKRMSRS**Q**GRVYYFNHITNASQFERPSG-COOH	4	987.72	987.75
23Qp	H_2N-KLPPGWEKRMSRS**Q**GRVYYFNHITNASQFERPSG-COOH	4	1035.28	1035.28
23X	H_2N-KLPPGWEKRMSRS**X**GRVYYFNHITNASQFERPSG-COOH	3	1316.00	1315.99
23Xp	H_2N-KLPPGWEKRMSRS**X**GRVYYFNHITNASQFERPSG-COOH	4	1037.78	1037.78
23Z	H_2N-KLPPGWEKRMSRS**Z**GRVYYFNHITNASQFERPSG-COOH	4	1006.01	1005.99
23Zp	H_2N-KLPPGWEKRMSRS**Z**GRVYYFNHITNASQFERPSG-COOH	4	1418.72	1418.74
23Nbp	H_2N-KLPPGWEKRMSRS**N**GRVYYFNHITNASQFERPSG-COOH	3	1438.74	1438.77
27Q	H_2N-KLPPGWEKRMSRSSGRV**Q**YFNHITNASQFERPSG-COOH	4	994.25	994.25
27Qp	H_2N-KLPPGWEKRMSRSSGRV**Q**YFNHITNASQFERPSG-COOH	4	1041.78	1041.80
27X	H_2N-KLPPGWEKRMSRSSGRV**X**YFNHITNASQFERPSG-COOH	4	993.75	993.75
27Xp	H_2N-KLPPGWEKRMSRSSGRV**X**YFNHITNASQFERPSG-COOH	4	1044.28	1044.28
27Z	H_2N-KLPPGWEKRMSRSSGRV**Z**YFNHITNASQFERPSG-COOH	4	1012.51	1012.50
27Zp	H_2N-KLPPGWEKRMSRSSGRV**Z**YFNHITNASQFERPSG-COOH	4	1070.79	1070.78
27Nbp	H_2N-KLPPGWEKRMSRSSGRV**N**YFNHITNASQFERPSG-COOH	3	1447.41	1447.44
29Q	H_2N-KLPPGWEKRMSRSSGRVYYFN**Q**ITNASQFERPSG-COOH	4	1003.26	1003.25
29Qp	H_2N-KLPPGWEKRMSRSSGRVYYFN**Q**ITNASQFERPSG-COOH	4	1050.79	1050.79
29X	H_2N-KLPPGWEKRMSRSSGRVYYFN**X**ITNASQFERPSG-COOH	3	1336.67	1336.67
29Xp	H_2N-KLPPGWEKRMSRSSGRVYYFN**X**ITNASQFERPSG-COOH	3	1404.04	1404.04
29Z	H_2N-KLPPGWEKRMSRSSGRVYYFN**Z**ITNASQFERPSG-COOH	4	1021.51	1021.52
29Zp	H_2N-KLPPGWEKRMSRSSGRVYYFN**Z**ITNASQFERPSG-COOH	4	1079.80	1079.82
29Nbp	H_2N-KLPPGWEKRMSRSSGRVYYFN**N**ITNASQFERPSG-COOH	3	1459.41	1459.42
32Q	H_2N-KLPPGWEKRMSRSSGRVYYFNHI**Q**NASQFERPSG-COOH	4	1006.76	1006.76
32Qp	H_2N-KLPPGWEKRMSRSSGRVYYFNHI**Q**NASQFERPSG-COOH	4	1054.29	1054.29
32X	H_2N-KLPPGWEKRMSRSSGRVYYFNHI**X**NASQFERPSG-COOH	4	1006.26	1006.25
32Xp	H_2N-KLPPGWEKRMSRSSGRVYYFNHI**X**NASQFERPSG-COOH	3	1408.72	1408.69
32Z	H_2N-KLPPGWEKRMSRSSGRVYYFNHI**Z**NASQFERPSG-COOH	4	1025.02	1025.03
32Zp	H_2N-KLPPGWEKRMSRSSGRVYYFNHI**Z**NASQFERPSG-COOH	4	1083.30	1083.30
32Nbp	H_2N-KLPPGWEKRMSRSSGRVYYFNHI**N**NASQFERPSG-COOH	4	1098.32	1098.31

[a] **N** = Asn-PEG; **N** = Asn-branched PEG; **Q** = Gln-PEG; **X** = Aha; **X** = Aha-PEG; **Z** = PrF; **Z** = PrF-PEG.

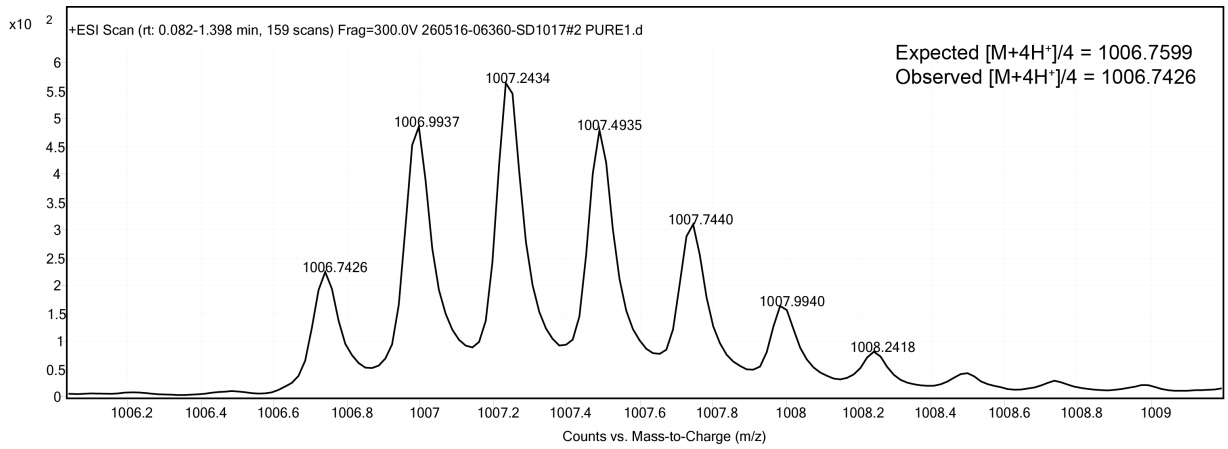


Figure S1. ESI-TOF spectrum for WW variant **16Q.**

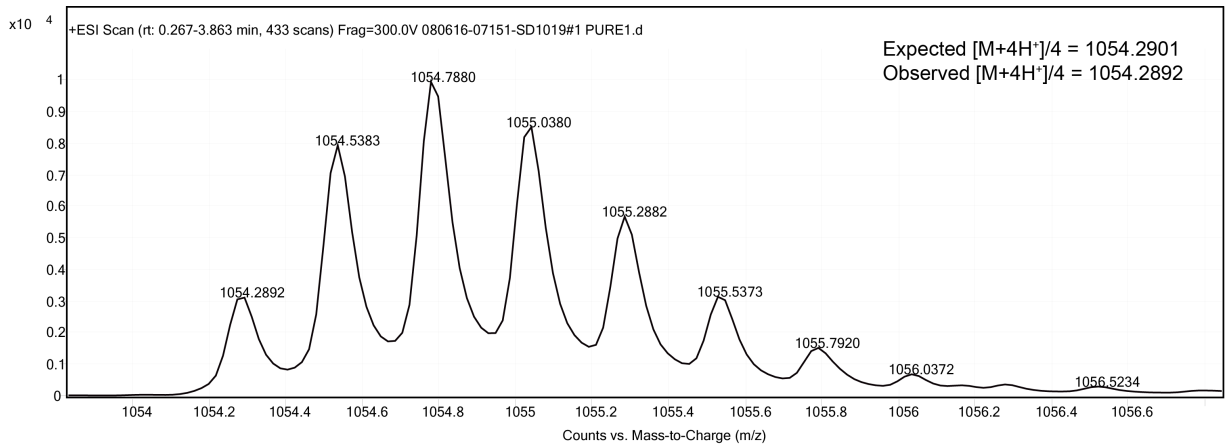


Figure S2. ESI-TOF spectrum for WW variant **16Qp.**

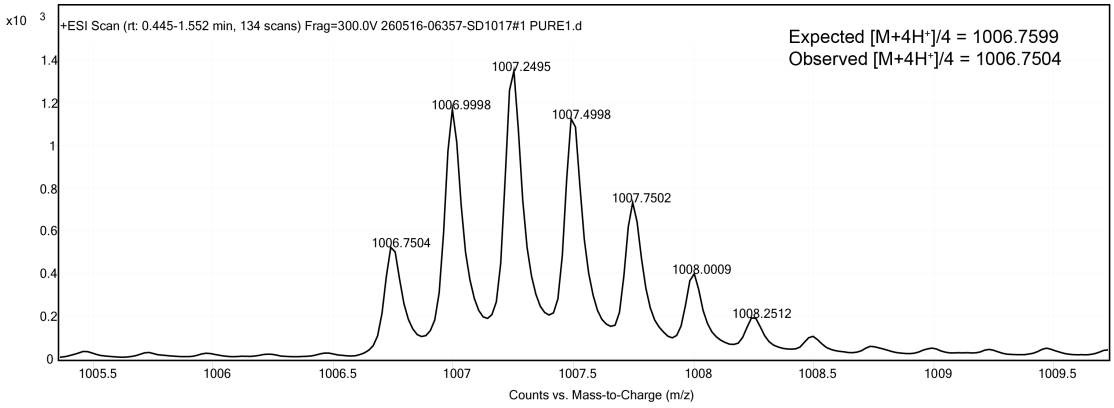


Figure S3. ESI-TOF spectrum for WW variant **18Q.**

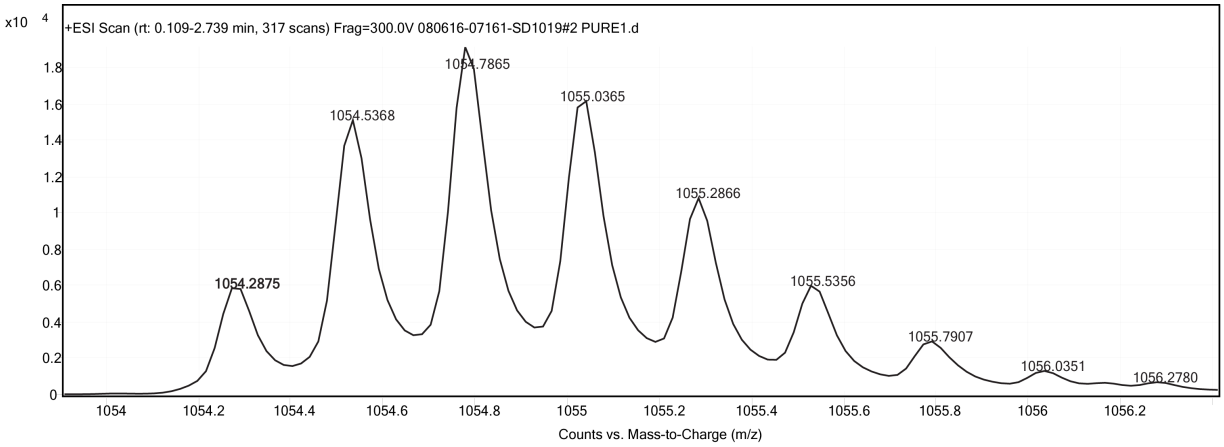


Figure S4. ESI-TOF spectrum for WW variant **18Qp.**

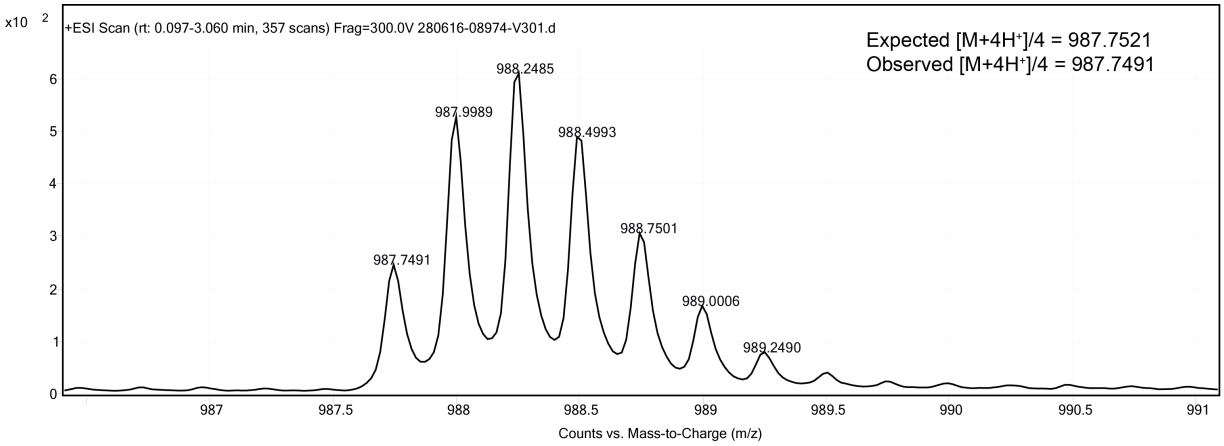


Figure S5. ESI-TOF spectrum for WW variant **23Q.**

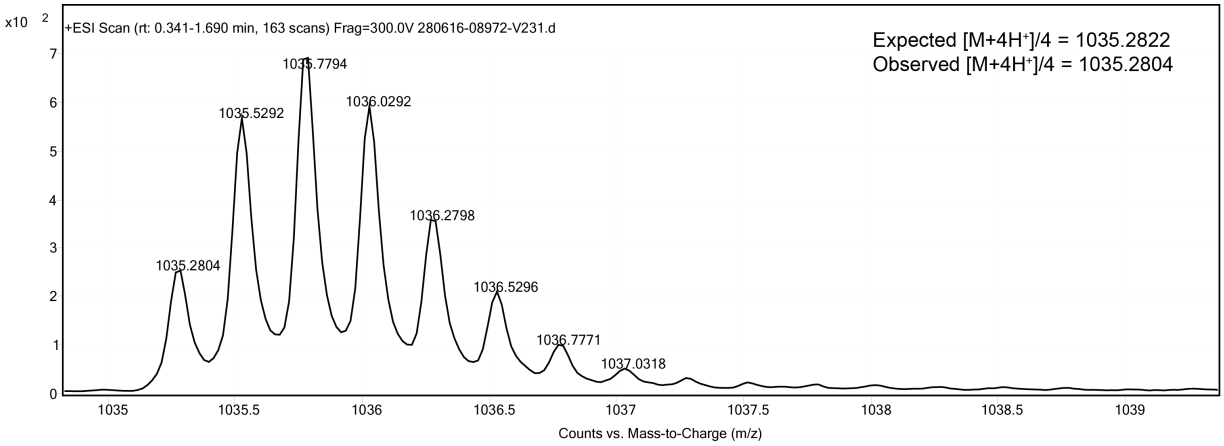


Figure S6. ESI-TOF spectrum for WW variant **23Qp.**

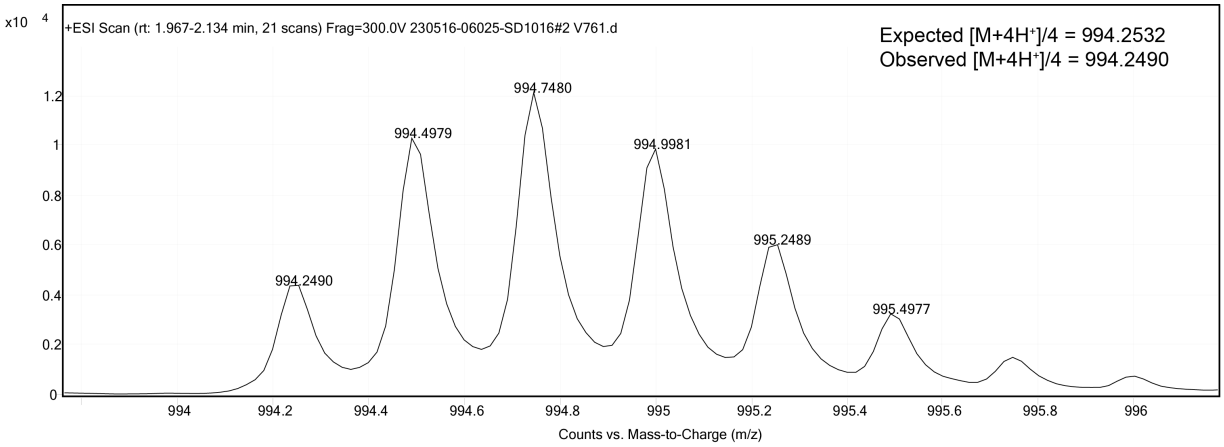


Figure S7. ESI-TOF spectrum for WW variant **27Q.**

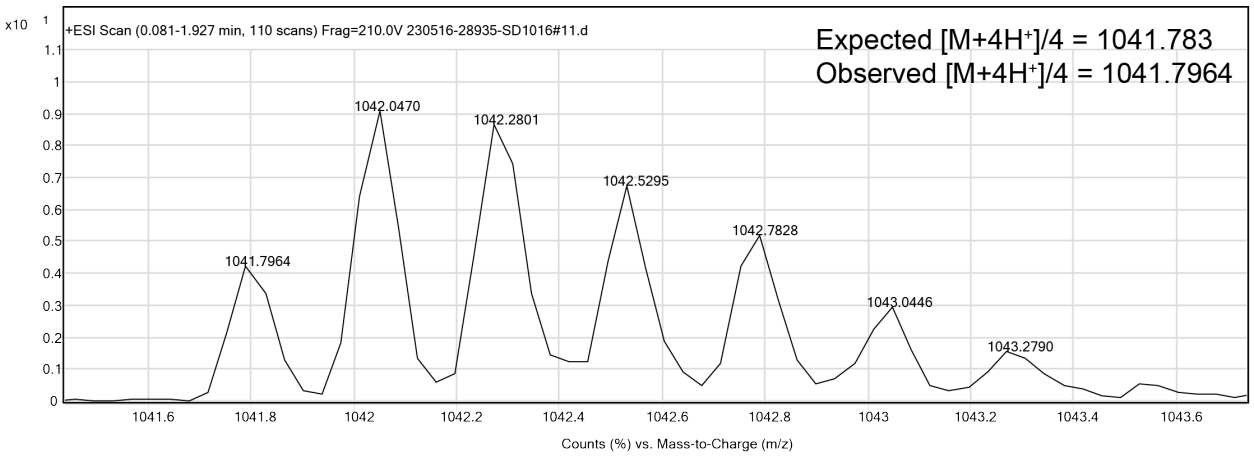


Figure S8. ESI-TOF spectrum for WW variant **27Qp.**

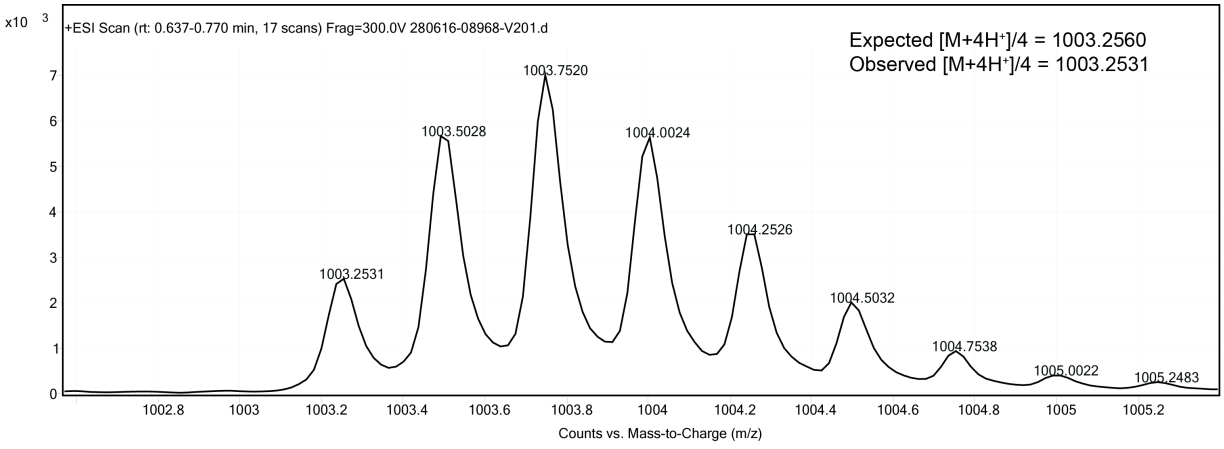


Figure S9. ESI-TOF spectrum for WW variant **29Q.**

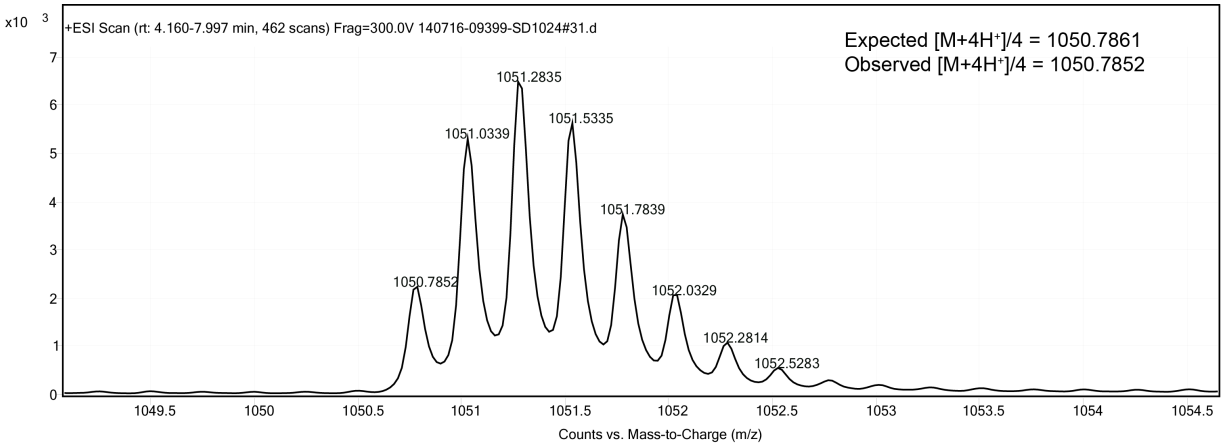


Figure S10. ESI-TOF spectrum for WW variant **29Qp.**

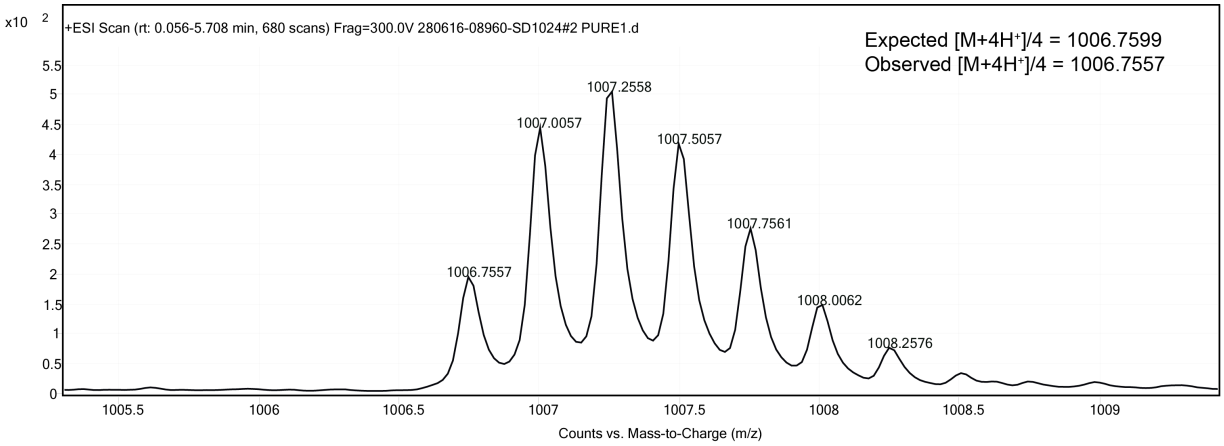


Figure S11. ESI-TOF spectrum for WW variant **32Q.**

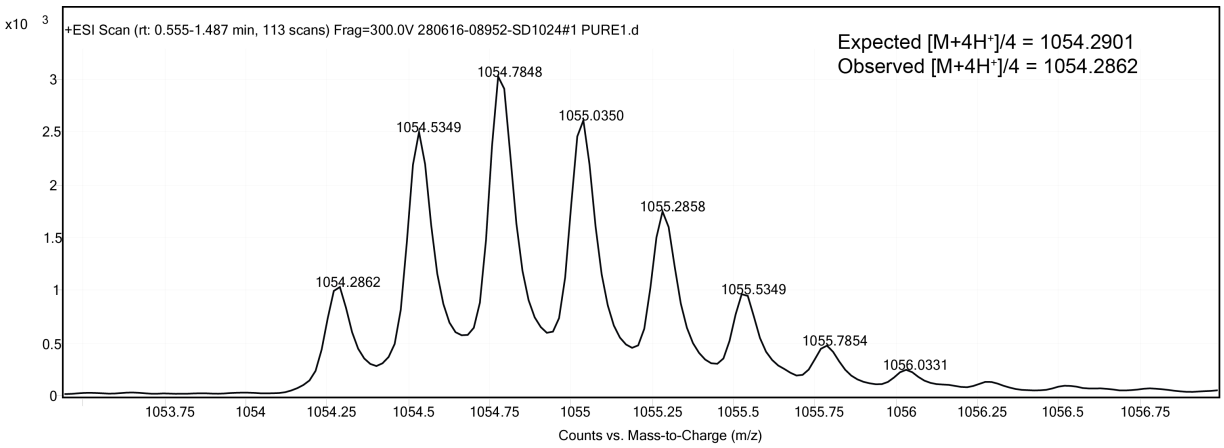


Figure S12. ESI-TOF spectrum for WW variant **32Qp.**

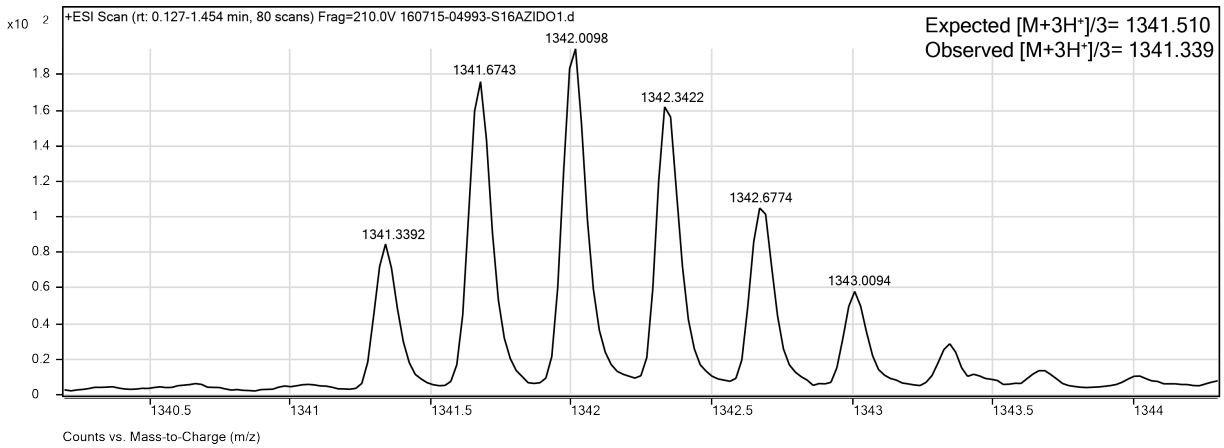


Figure S13. ESI-TOF spectrum for WW variant **16X**.

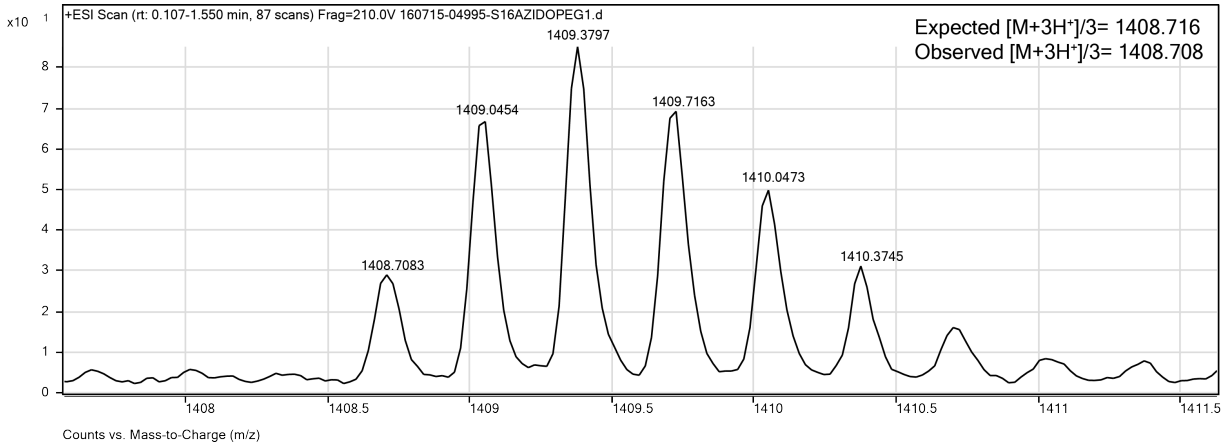


Figure S14. ESI-TOF spectrum for WW variant **16Xp.**

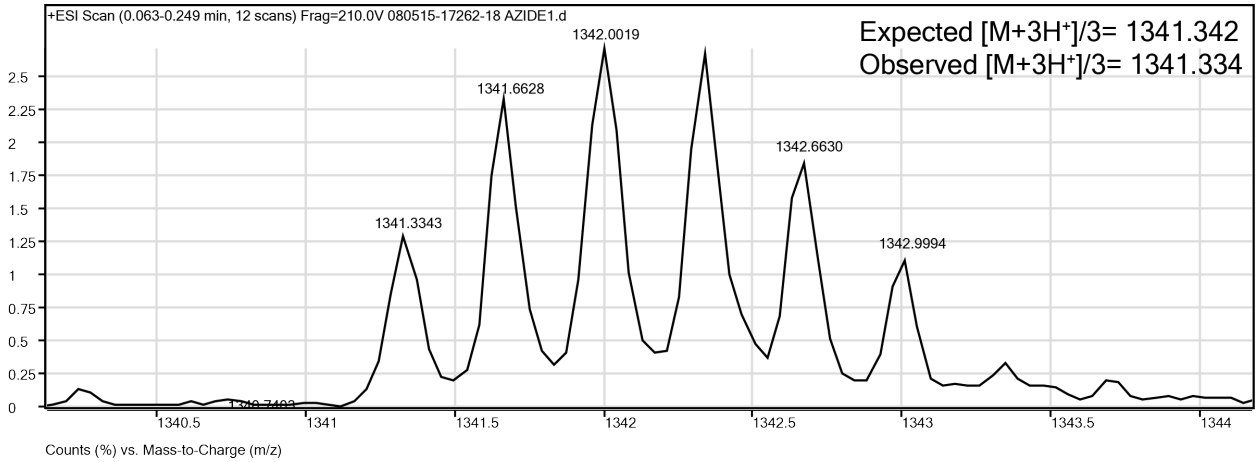


Figure S15. ESI-TOF spectrum for WW variant **18X**.

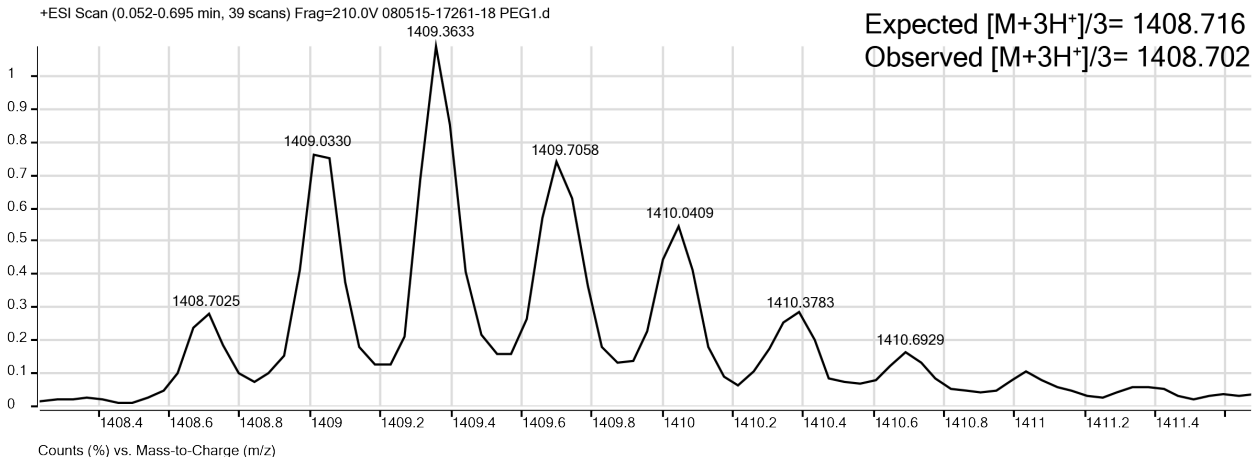


Figure S16. ESI-TOF spectrum for WW variant **18Xp.**

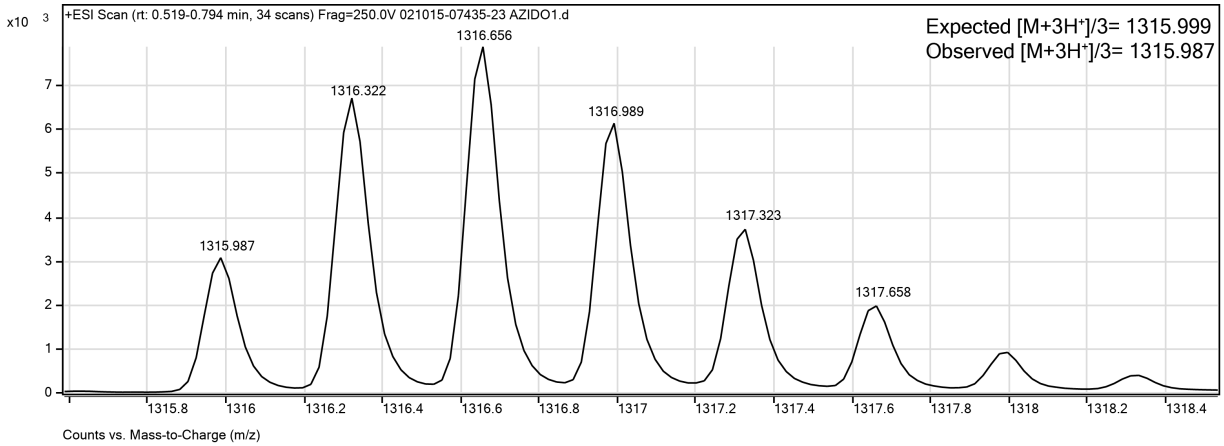


Figure S17. ESI-TOF spectrum for WW variant **23X**.

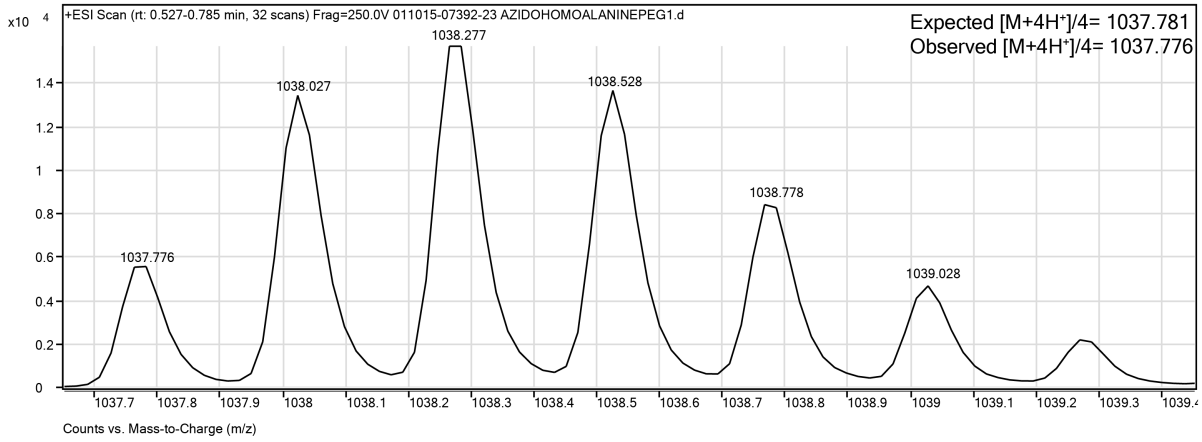


Figure S18. ESI-TOF spectrum for WW variant **23Xp**.

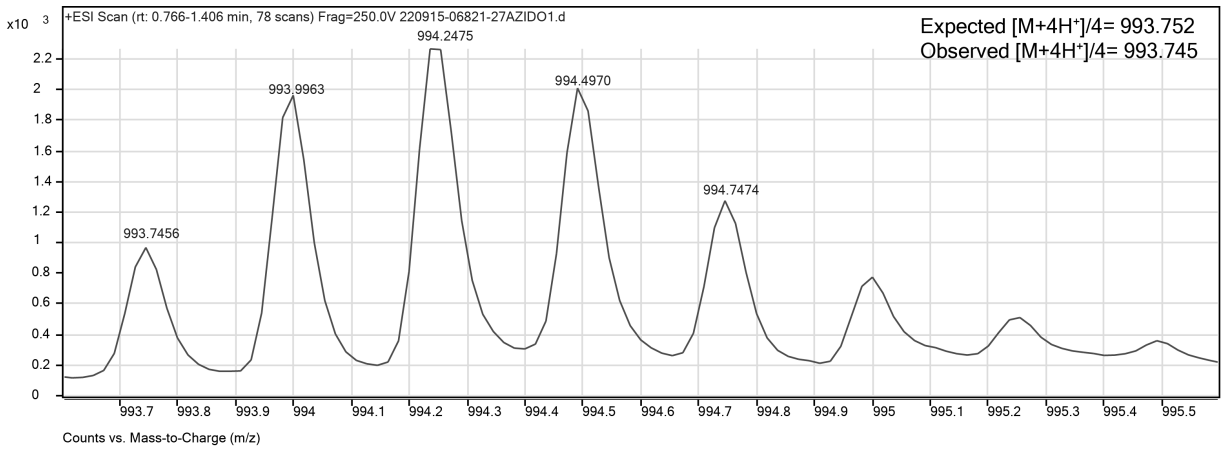


Figure S191. ESI-TOF spectrum for WW variant **27X**.

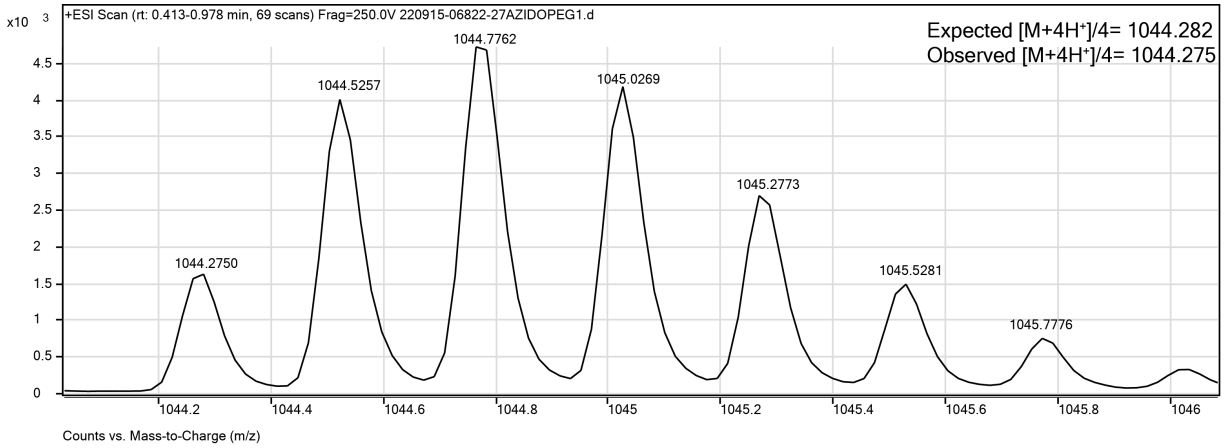


Figure S20. ESI-TOF spectrum for WW variant **27Xp**.

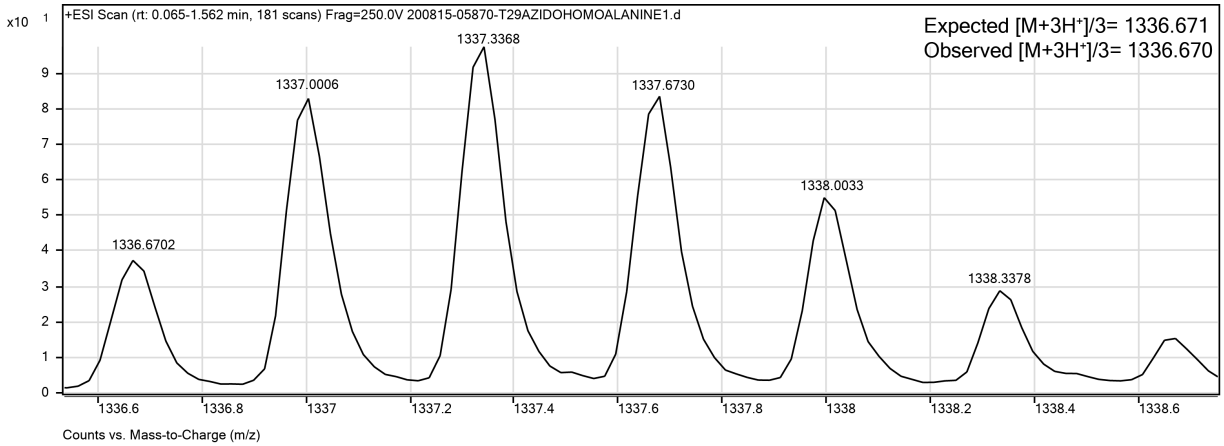


Figure S20. ESI-TOF spectrum for WW variant **29X**.

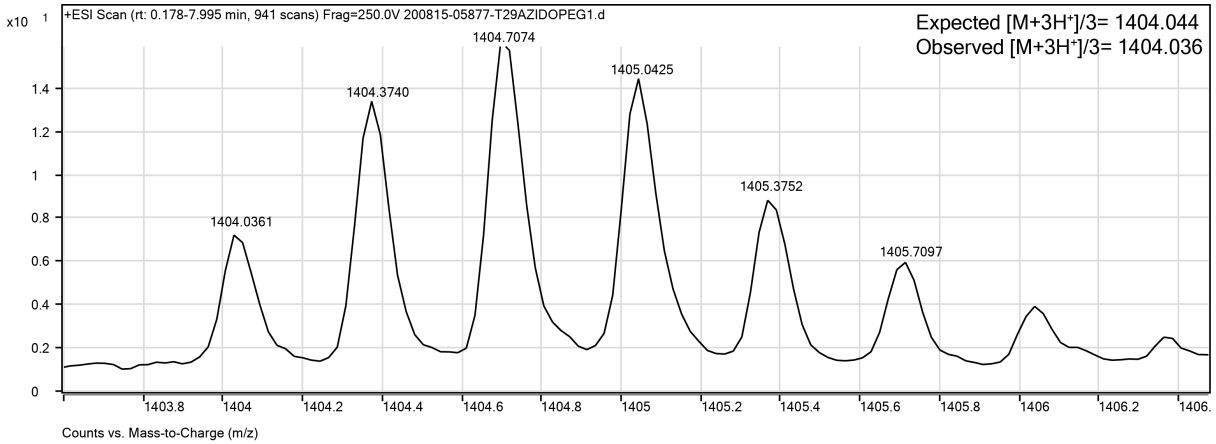


Figure S21. ESI-TOF spectrum for WW variant **29Xp**.

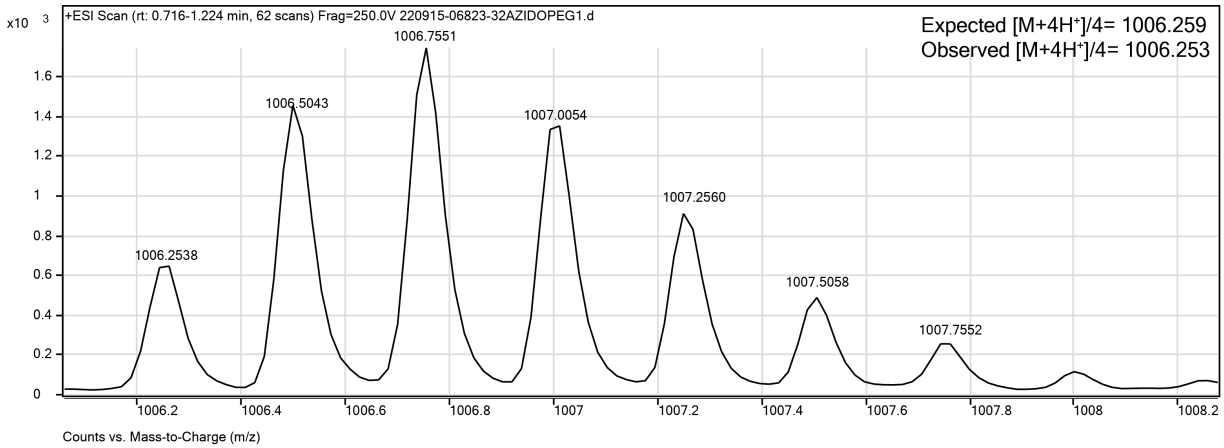


Figure S22. ESI-TOF spectrum for WW variant **32X**.

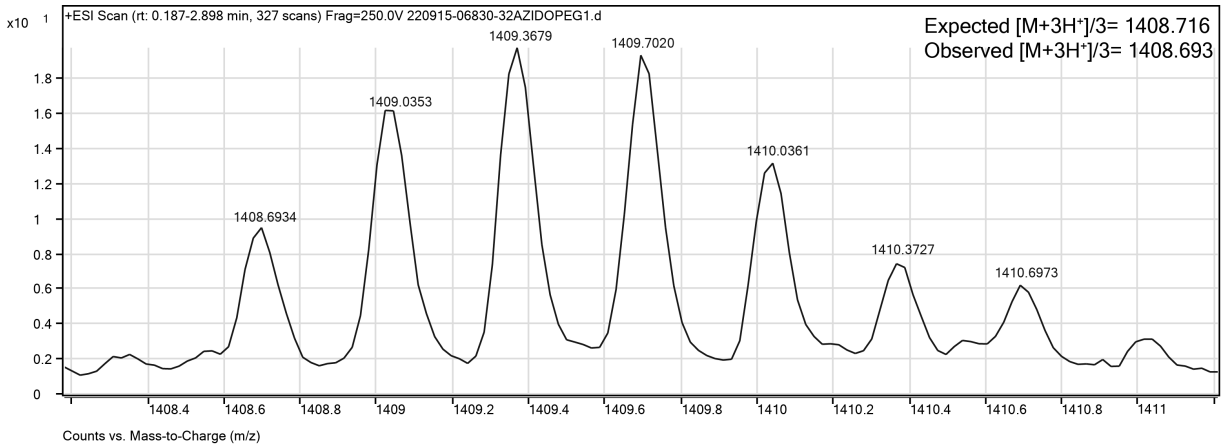


Figure S23. ESI-TOF spectrum for WW variant **32Xp**.

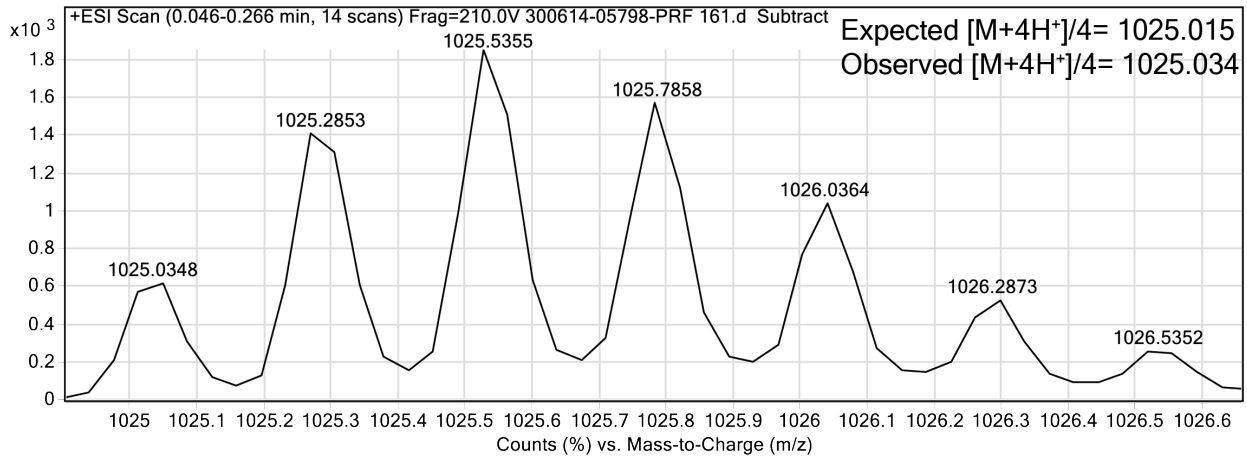


Figure S24. ESI-TOF spectrum for WW variant **16Z**.

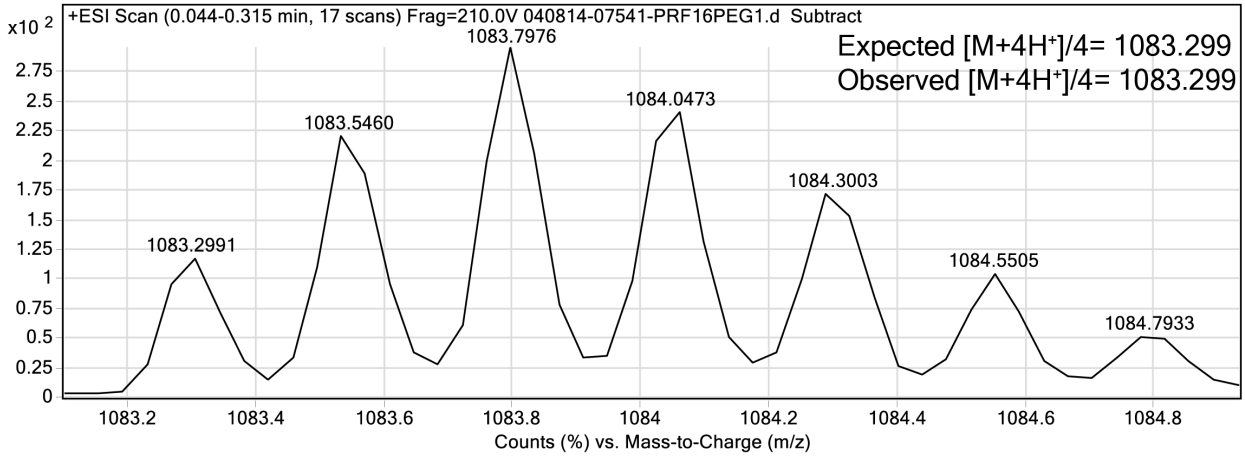


Figure S25. ESI-TOF spectrum for WW variant **16Zp**.

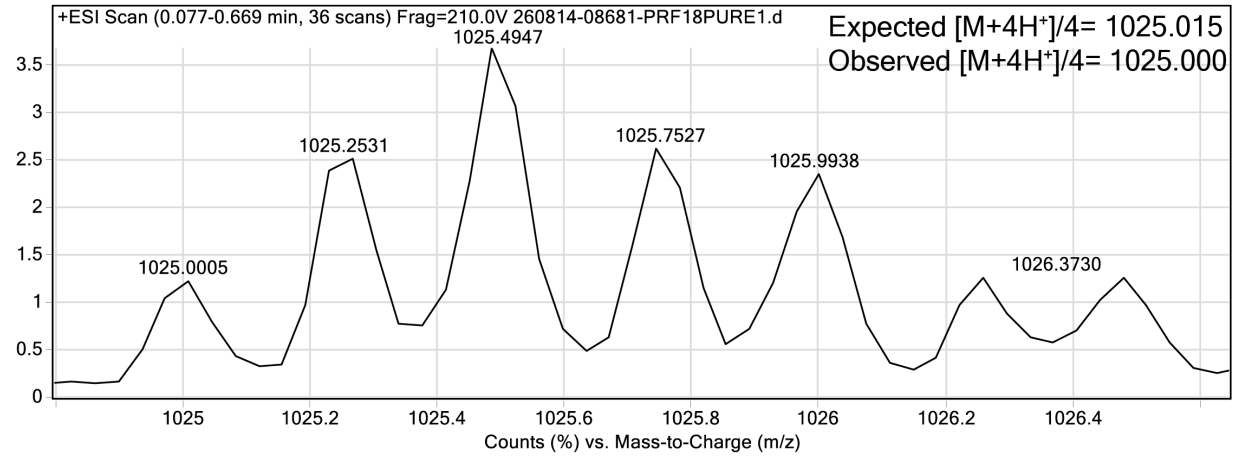


Figure S26. ESI-TOF spectrum for WW variant **18Z**.

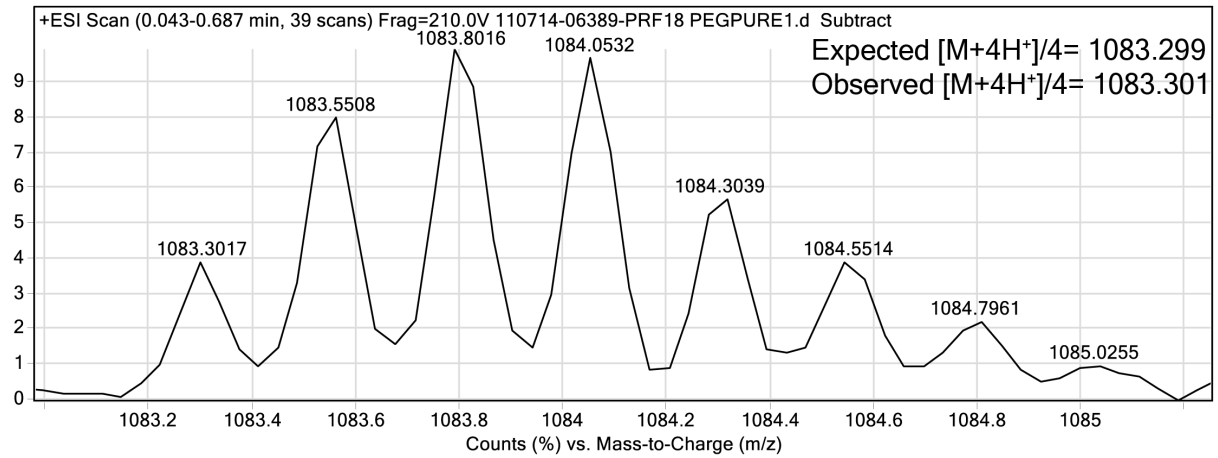


Figure S27. ESI-TOF spectrum for WW variant **18Zp**.

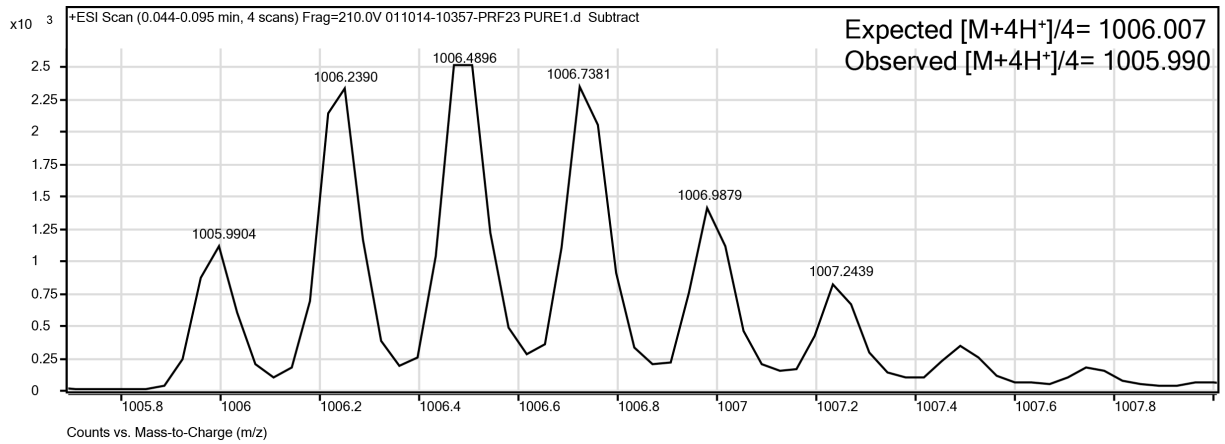


Figure S28. ESI-TOF spectrum for WW variant **23Z**.

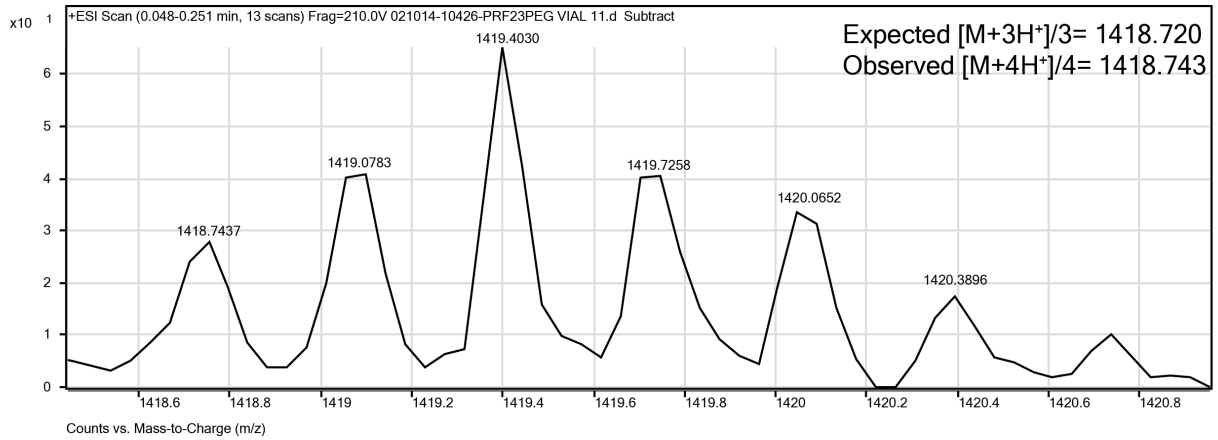


Figure S29. ESI-TOF spectrum for WW variant **23Zp**.

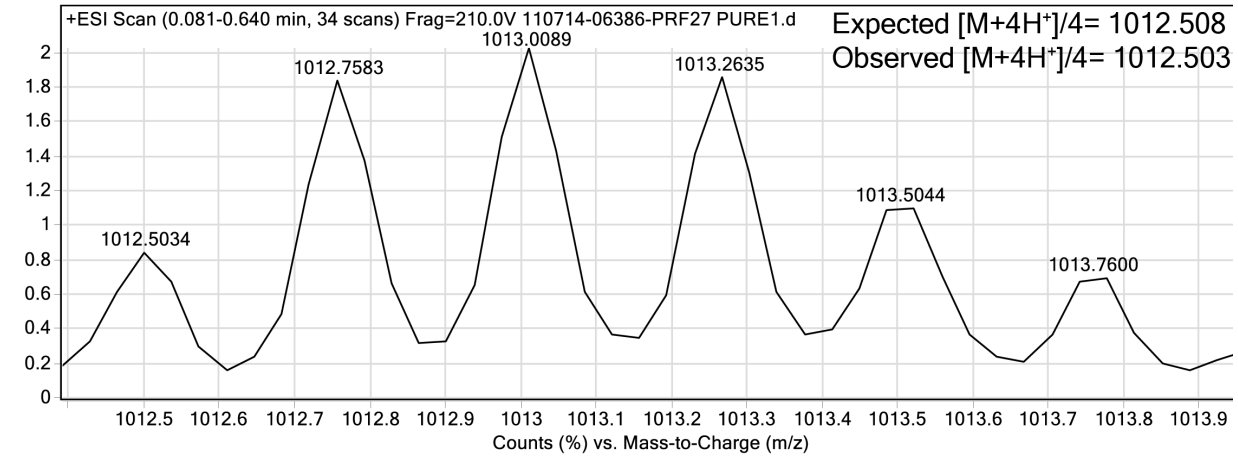


Figure S30. ESI-TOF spectrum for WW variant **27Z**.

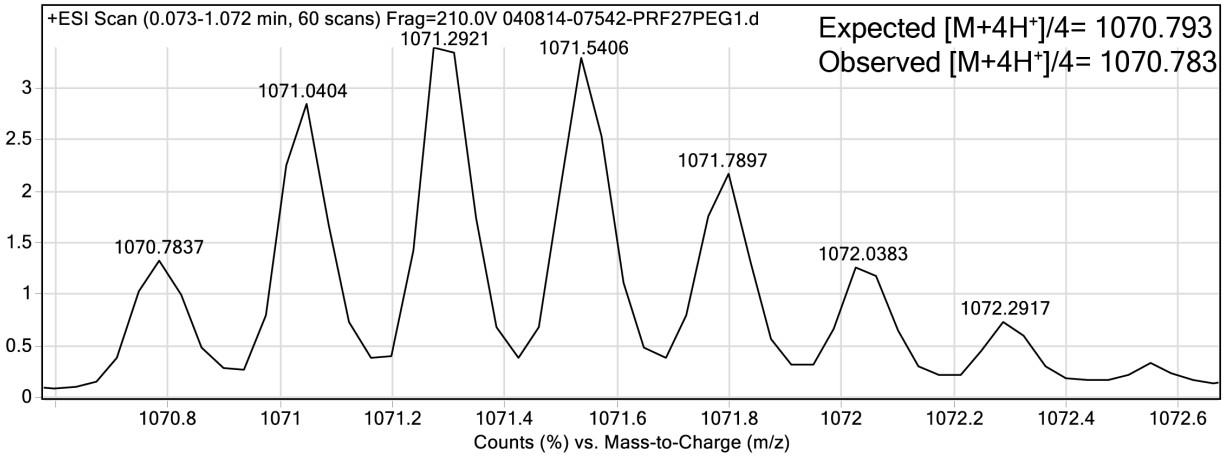


Figure S31. ESI-TOF spectrum for WW variant **27Zp**.

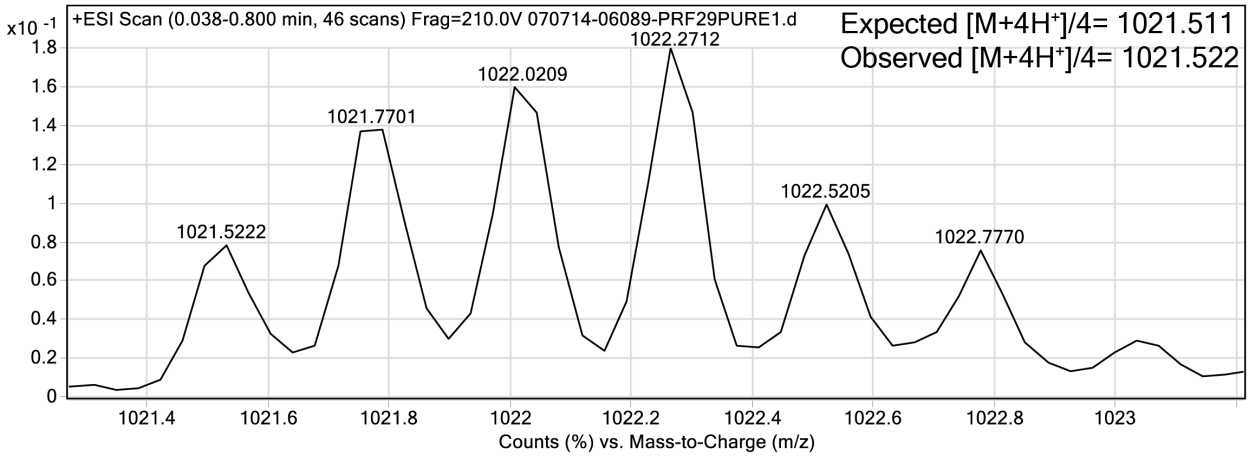


Figure S32. ESI-TOF spectrum for WW variant **29Z**.

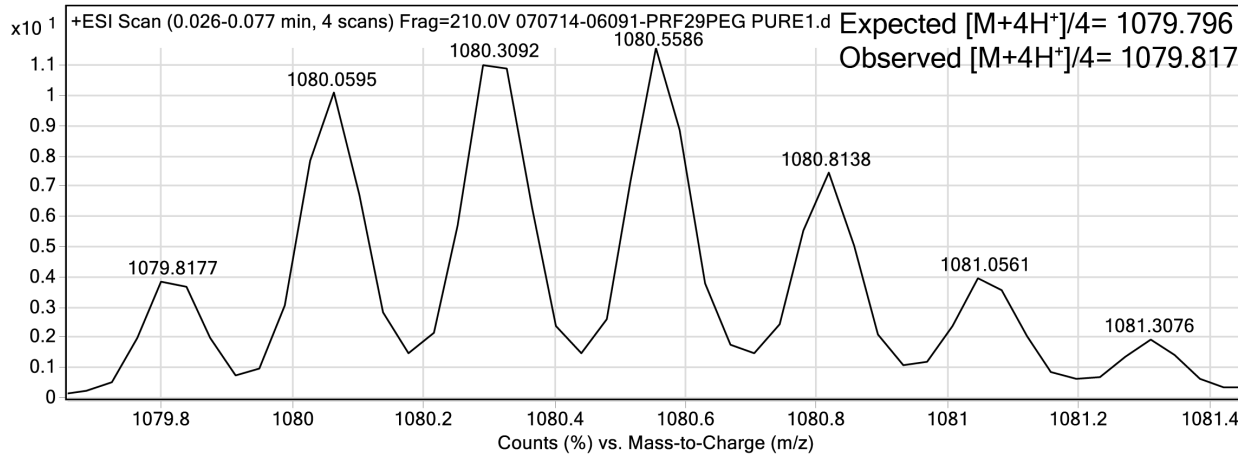


Figure S33. ESI-TOF spectrum for WW variant **29Zp**.

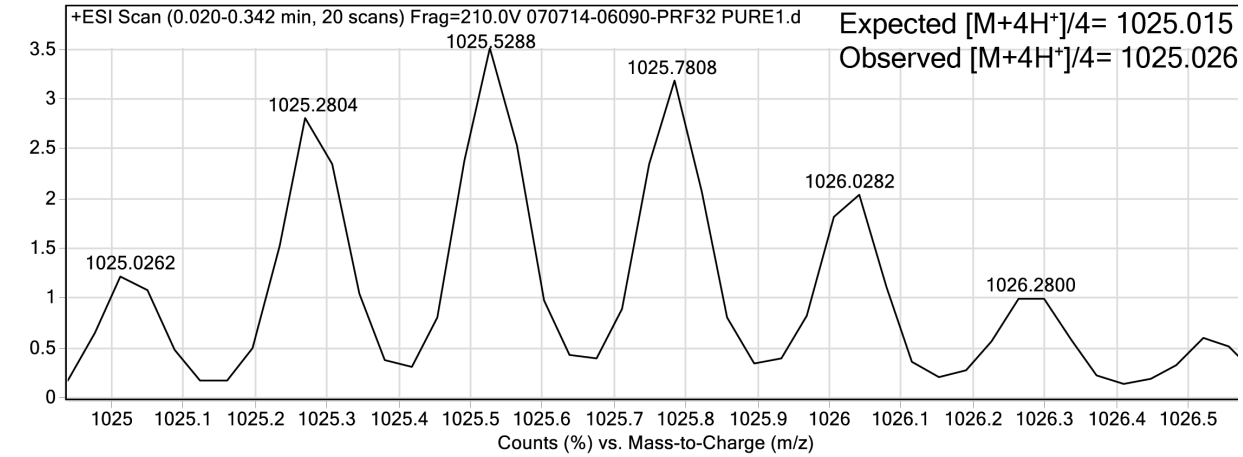


Figure S34. ESI-TOF spectrum for WW variant **32Z**.

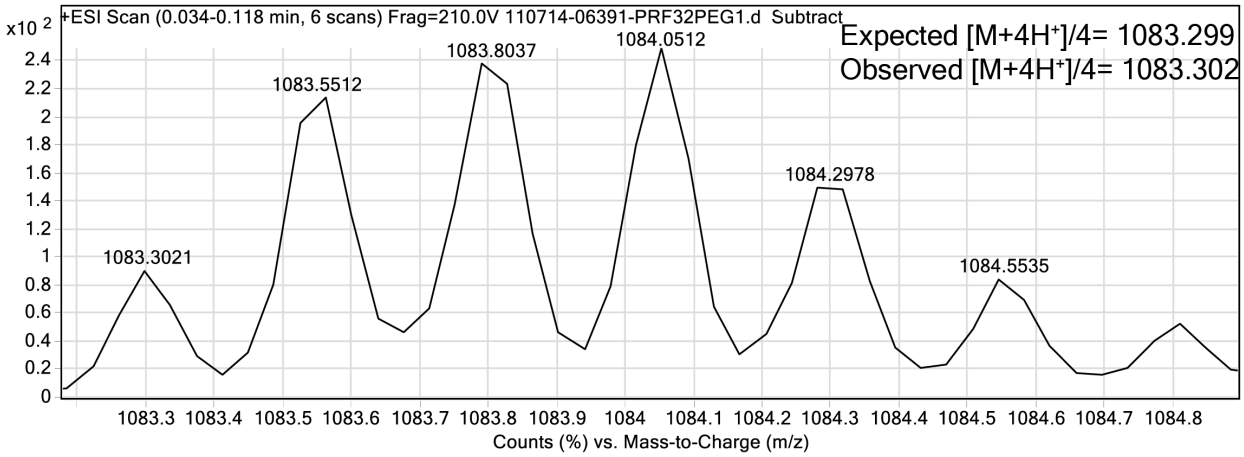


Figure S35. ESI-TOF spectrum for WW variant **32Zp**.

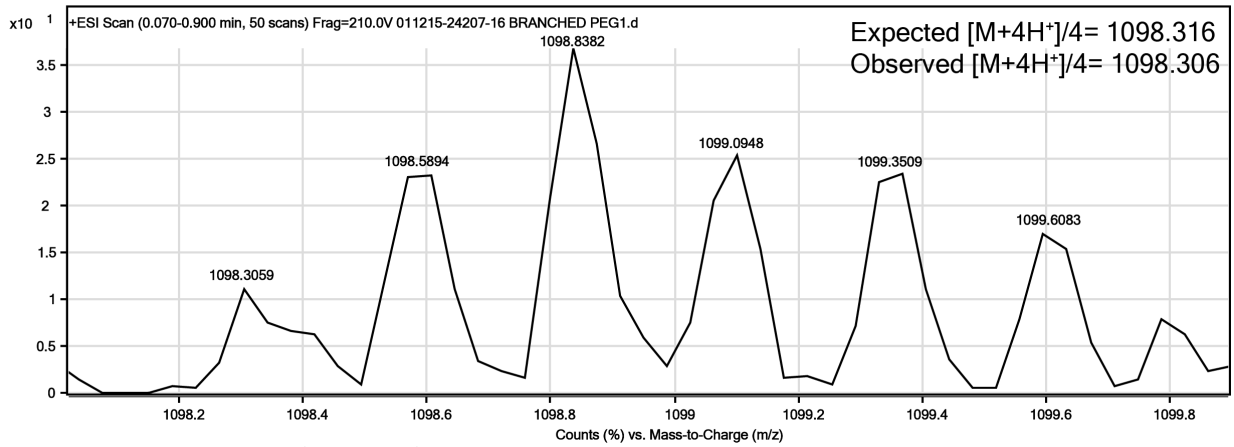


Figure S36. ESI-TOF spectrum for WW variant **16Nbp**.

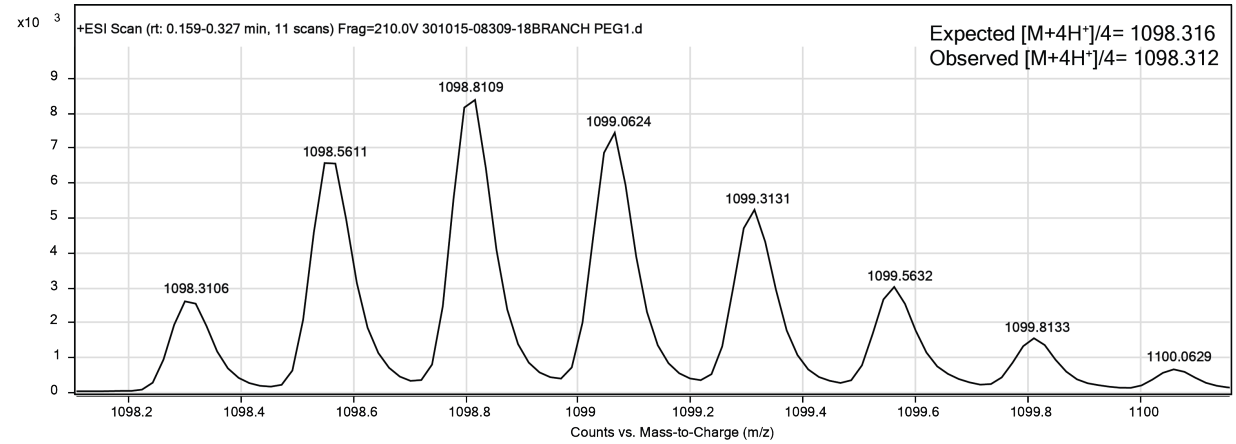


Figure S37. ESI-TOF spectrum for WW variant **18Nbp**.

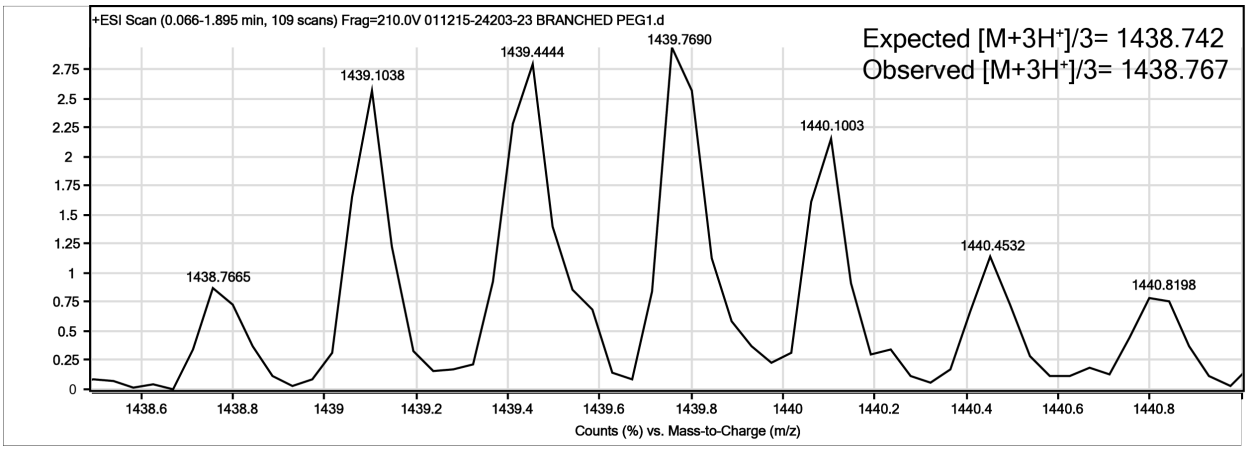


Figure S38. ESI-TOF spectrum for WW variant **23Nbp**.

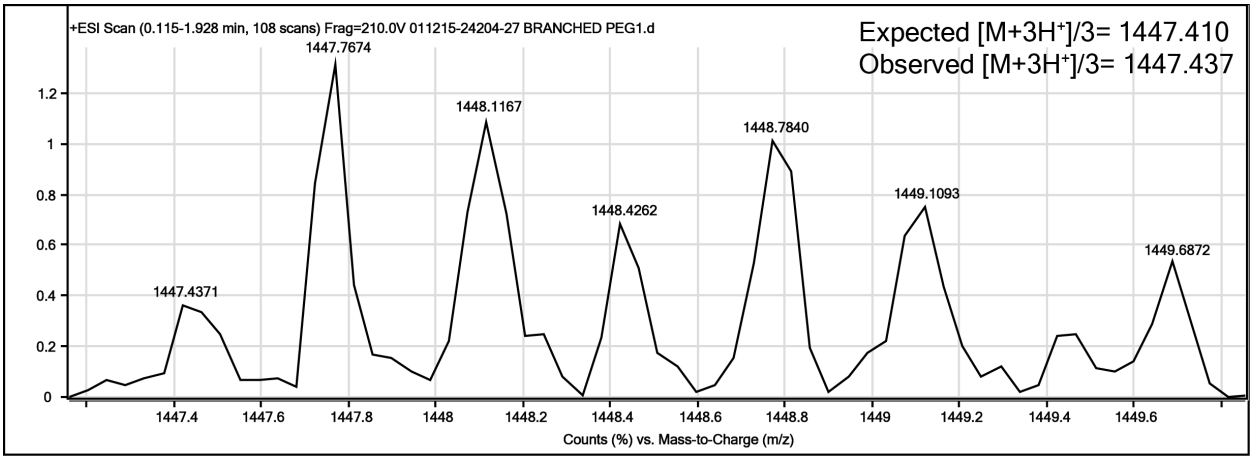


Figure S39. ESI-TOF spectrum for WW variant **27Nbp**.

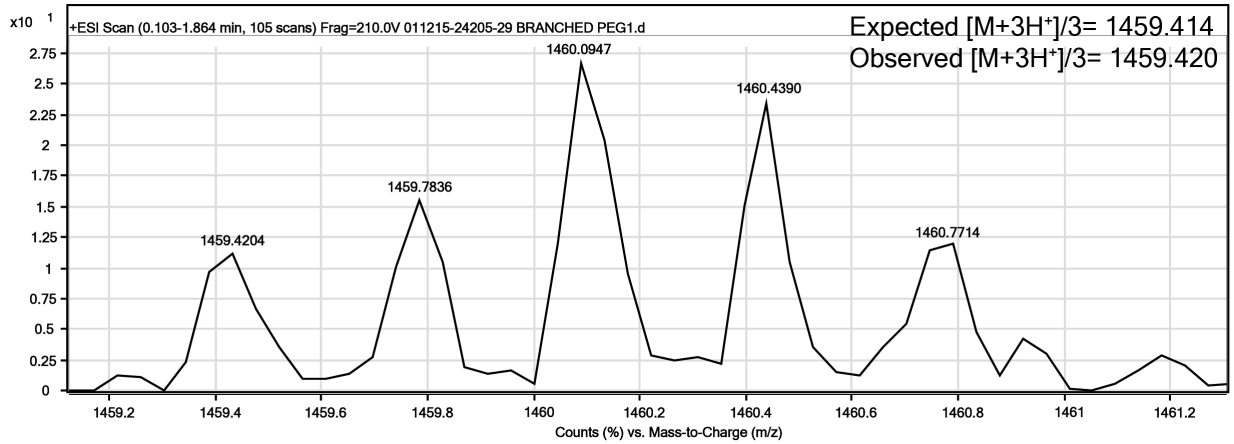


Figure S40. ESI-TOF spectrum for WW variant **29Nbp**.

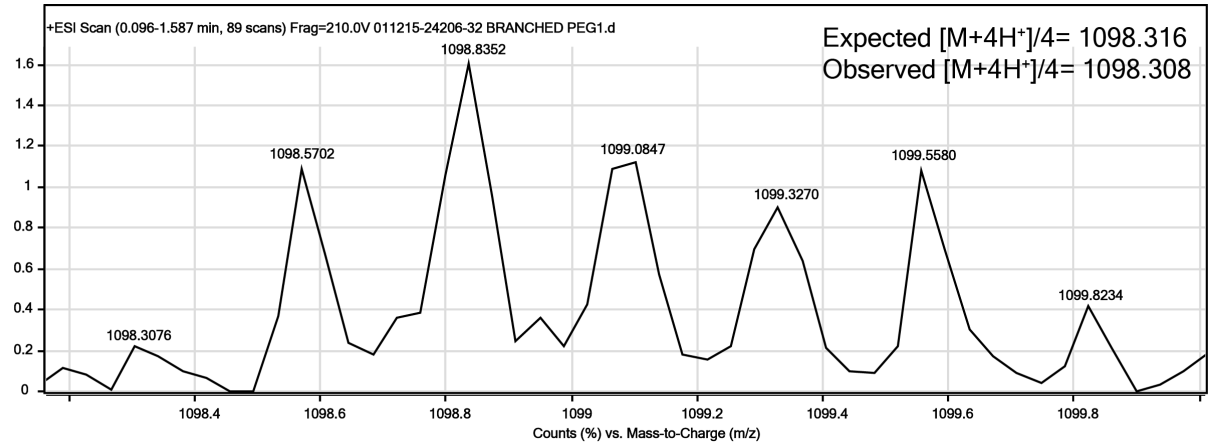


Figure S41. ESI-TOF spectrum for WW variant **32Nbp**.

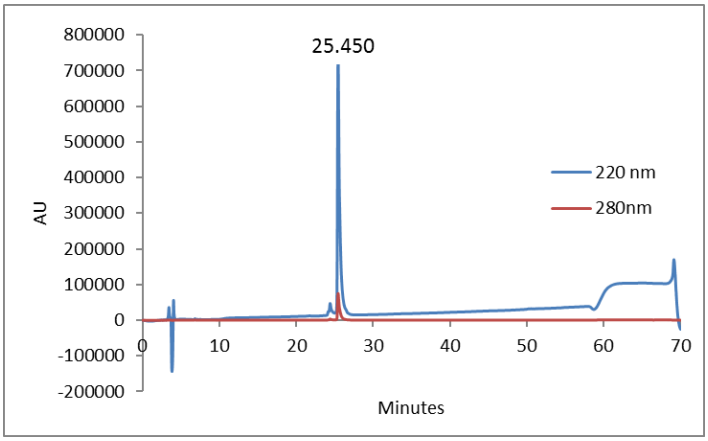


Figure S42. Analytical HPLC Data for WW variant **16Q**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

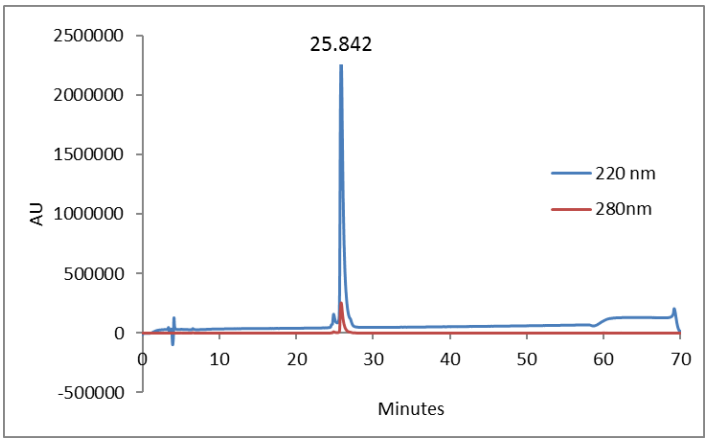


Figure S43. Analytical HPLC Data for WW variant **16Qp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

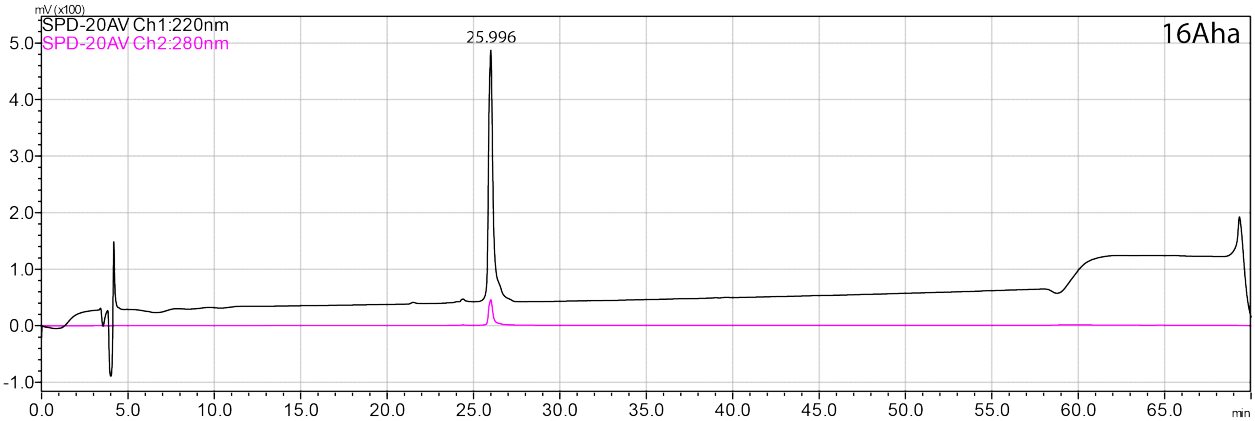


Figure S44. Analytical HPLC Data for WW variant **16X**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

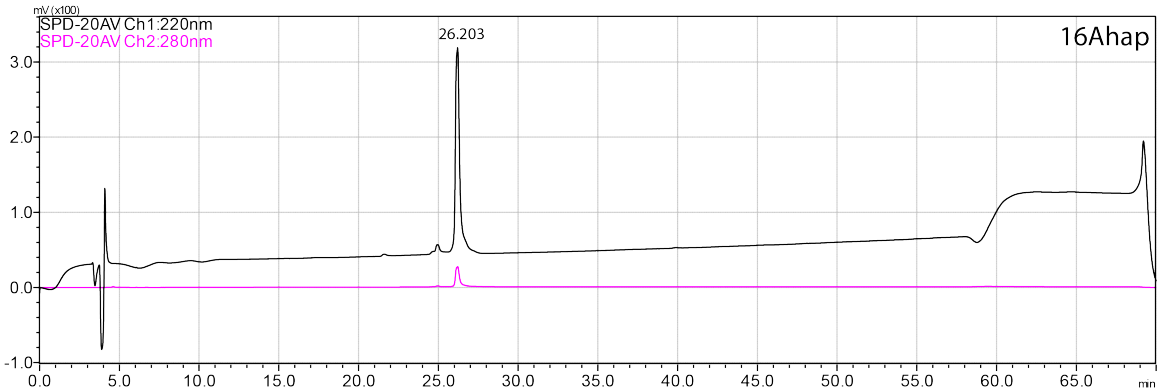


Figure S45. Analytical HPLC Data for WW variant **16Xp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

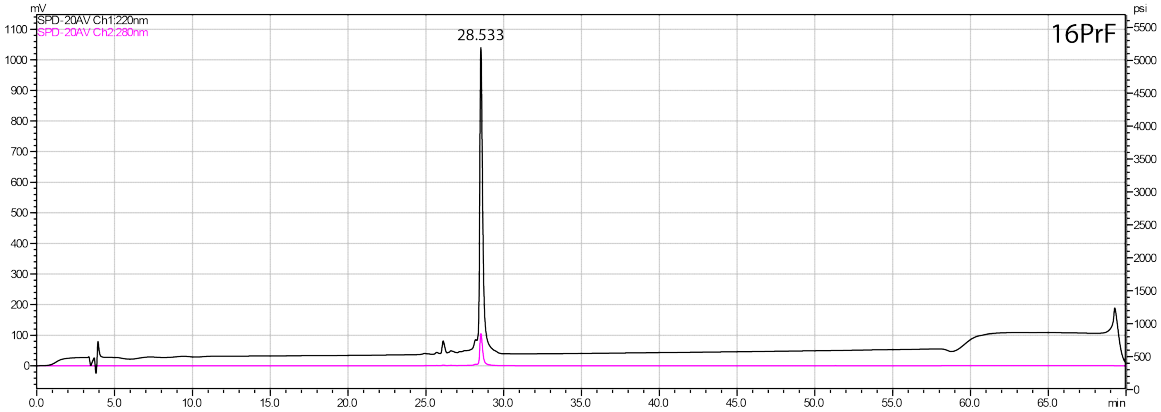


Figure S46. Analytical HPLC Data for WW variant **16Z**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min

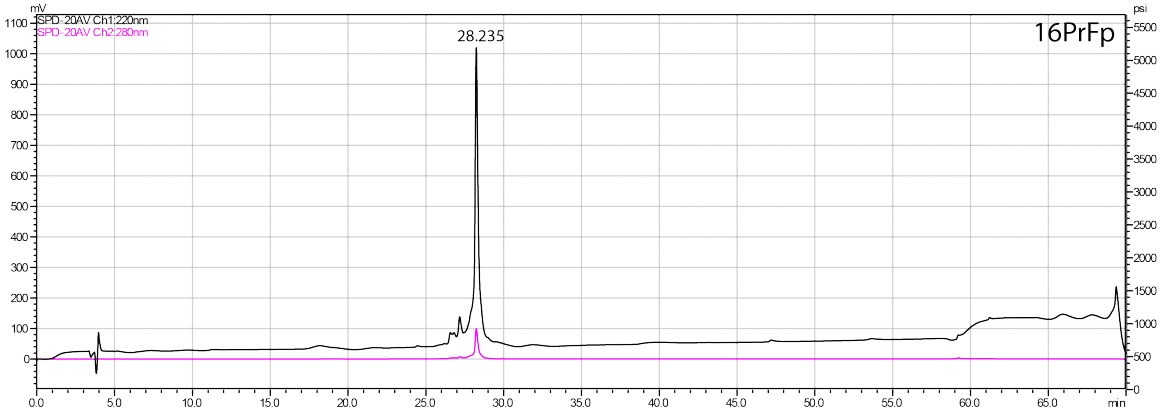


Figure S47. Analytical HPLC Data for WW variant **16Zp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

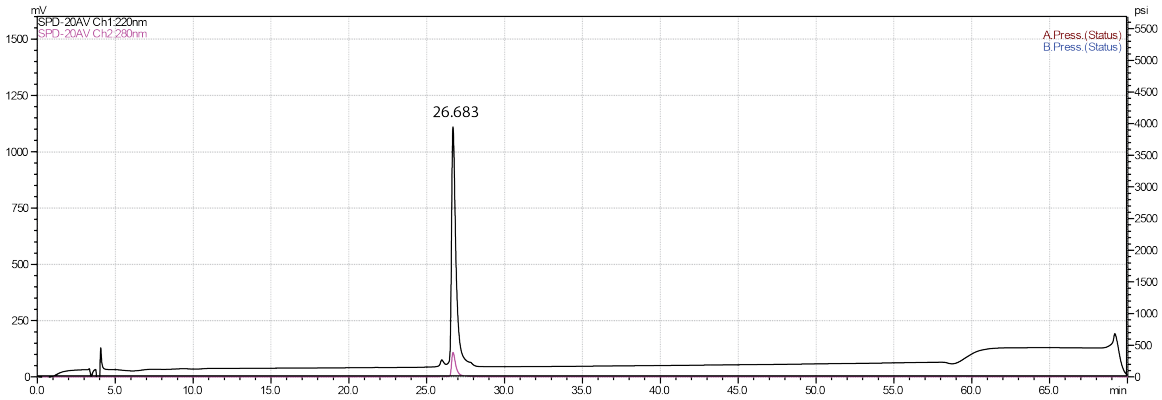


Figure S48. Analytical HPLC Data for WW variant **16Nbp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

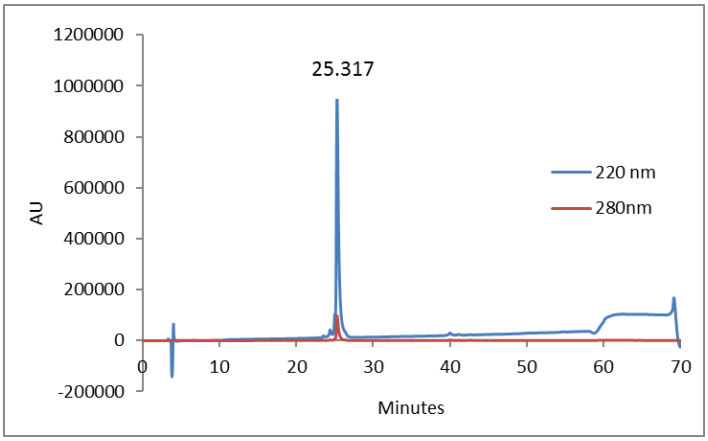


Figure S49. Analytical HPLC Data for WW variant **18Q**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

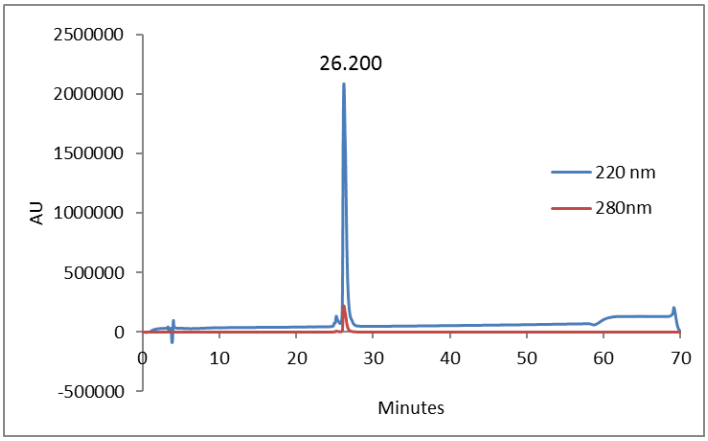


Figure S50. Analytical HPLC Data for WW variant **18Qp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

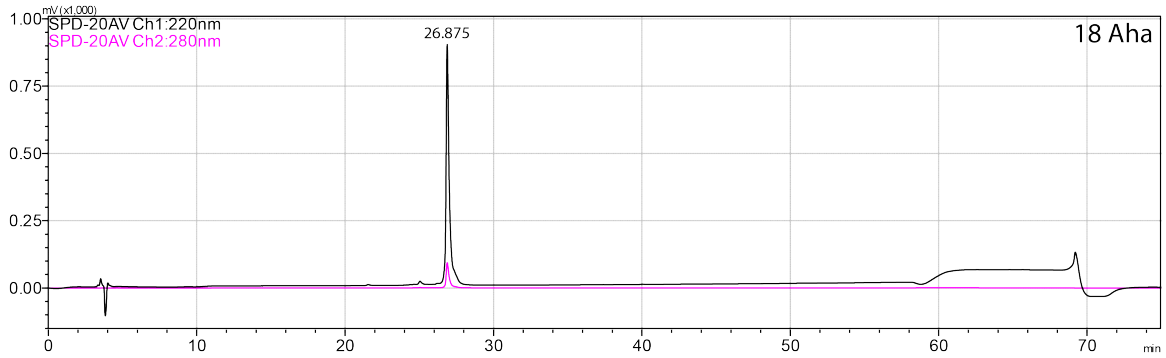


Figure S51. Analytical HPLC Data for WW variant **18X**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

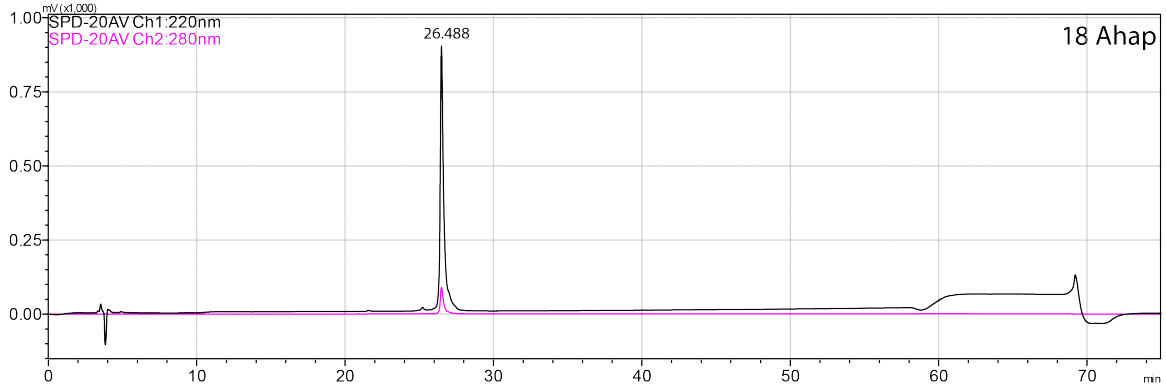


Figure S52. Analytical HPLC Data for WW variant **18Xp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

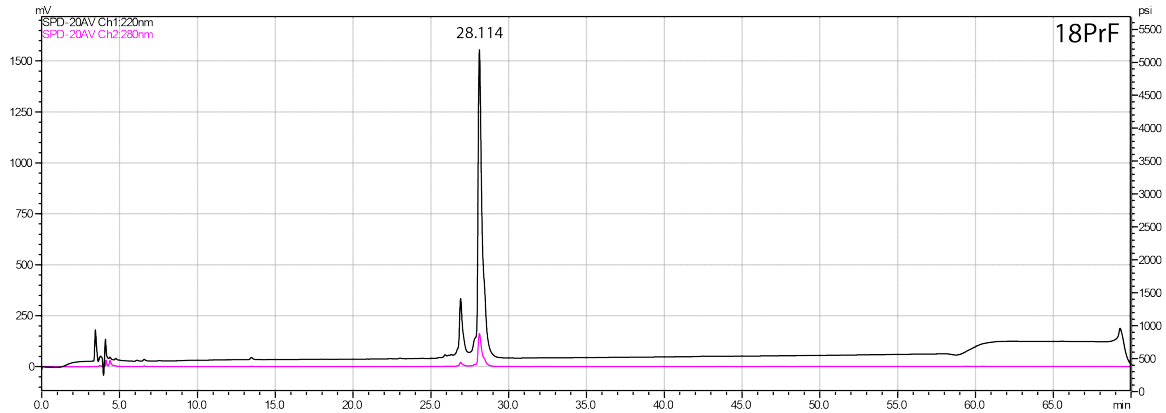


Figure S53. Analytical HPLC Data for WW variant **18Z**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

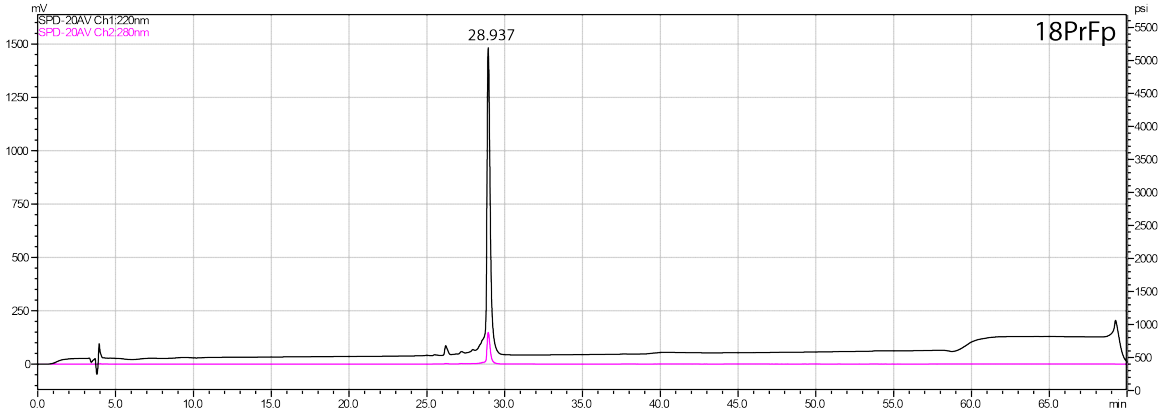


Figure S54. Analytical HPLC Data for WW variant **18Zp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

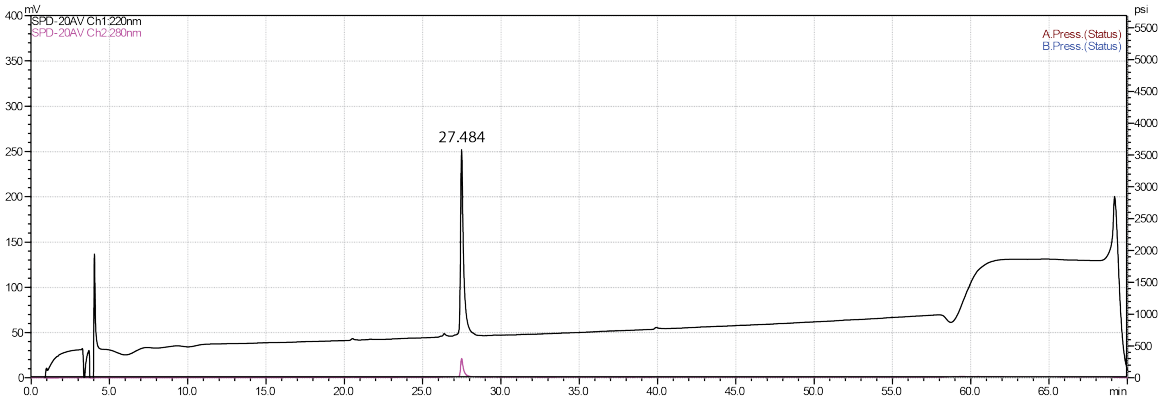


Figure S55. Analytical HPLC Data for WW variant **18Nbp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

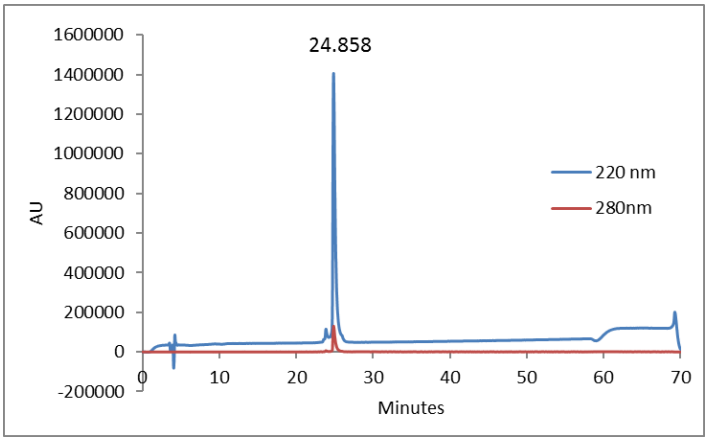


Figure S57. Analytical HPLC Data for WW variant **23Q**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

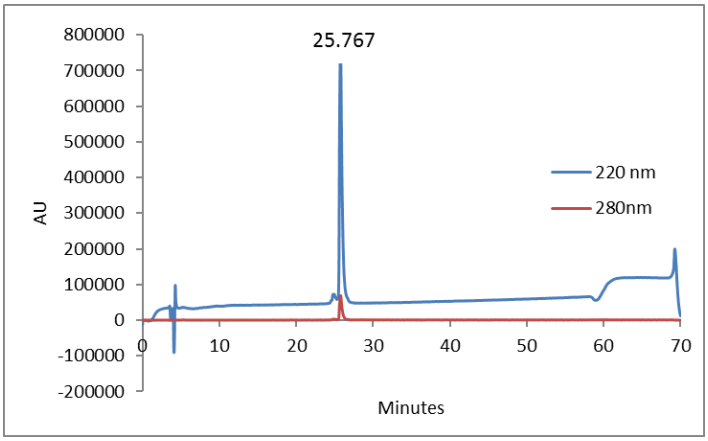


Figure S58. Analytical HPLC Data for WW variant **23Qp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

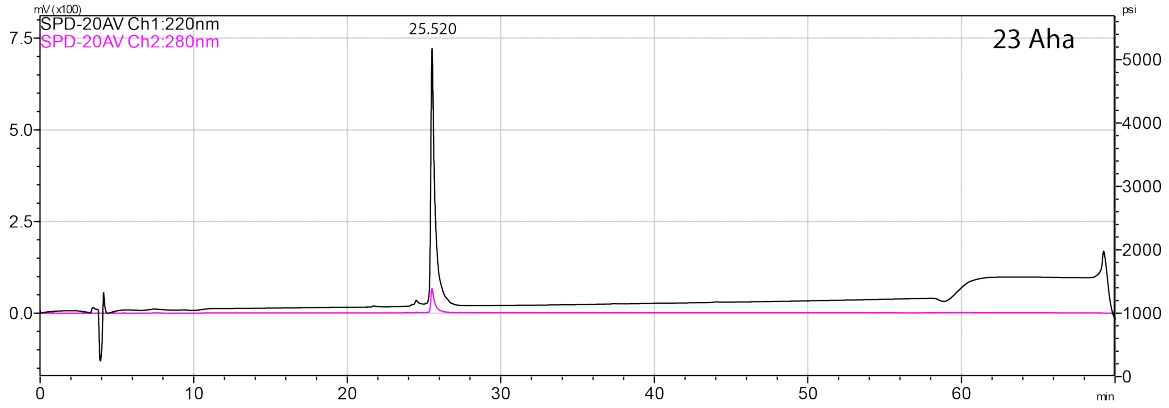


Figure S60. Analytical HPLC Data for WW variant **23X**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

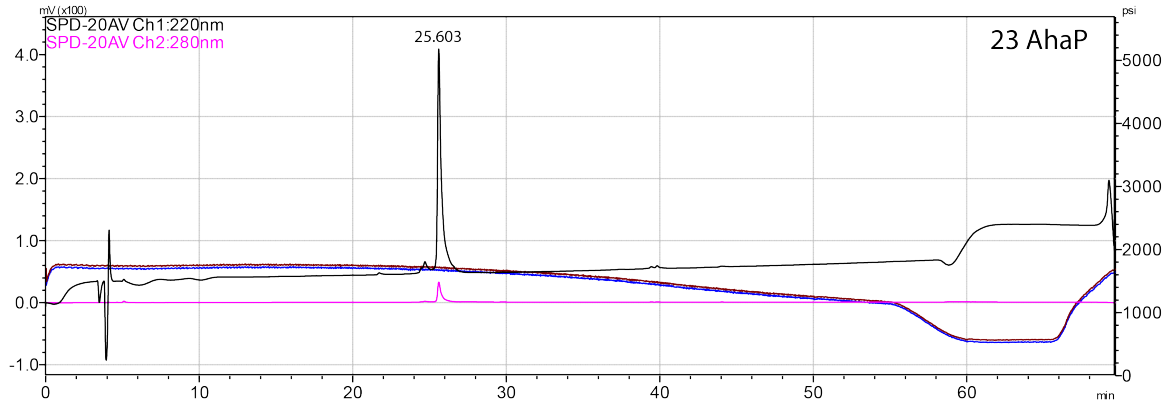


Figure S61. Analytical HPLC Data for WW variant **23Xp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

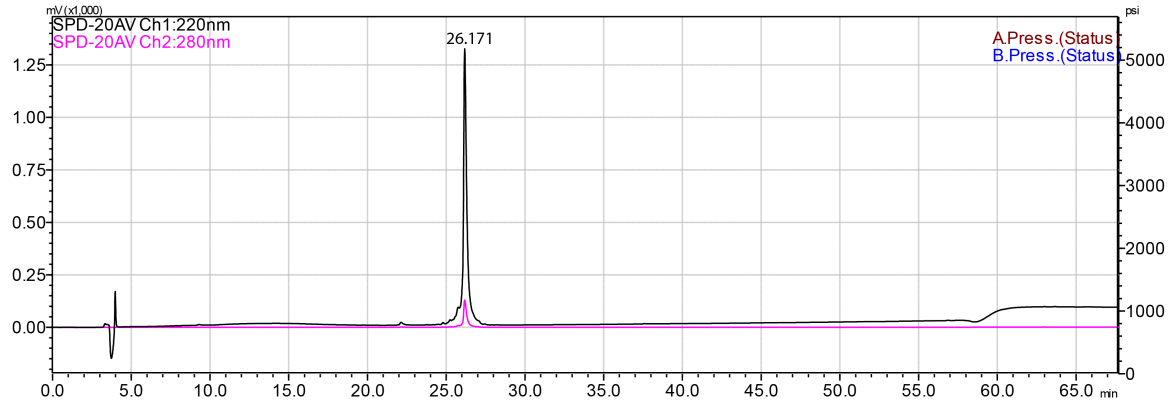


Figure S62. Analytical HPLC Data for WW variant **23Z**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

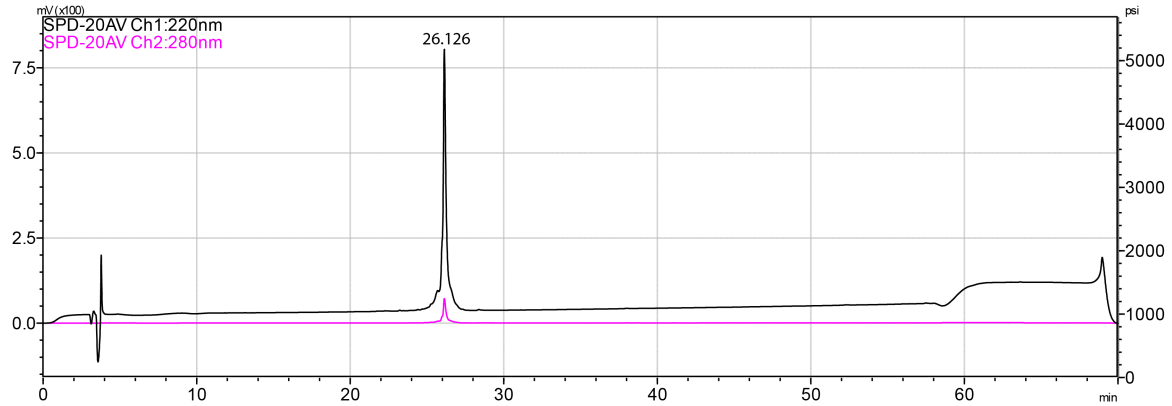


Figure S63. Analytical HPLC Data for WW variant **23Zp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

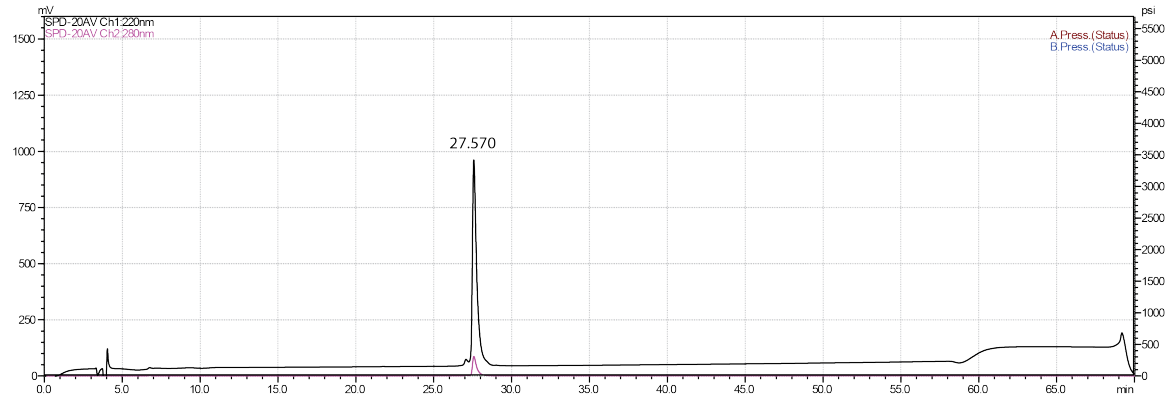


Figure S64. Analytical HPLC Data for WW variant **23Nbp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

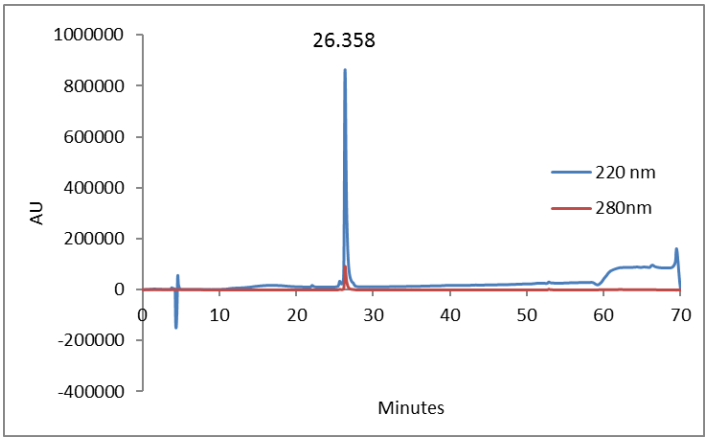


Figure S65. Analytical HPLC Data for WW variant **27Q**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

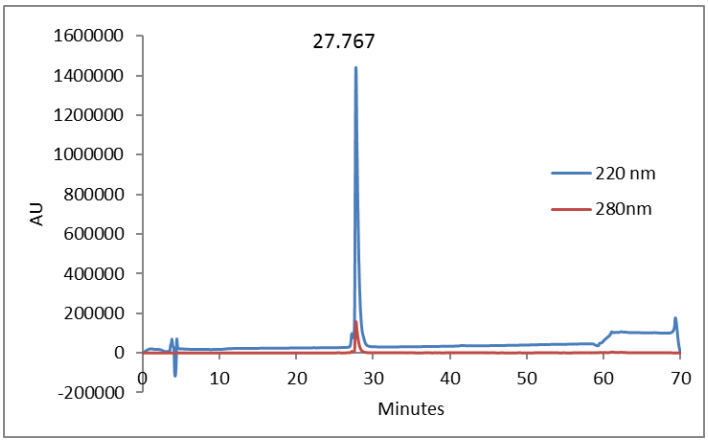


Figure S66. Analytical HPLC Data for WW variant **27Qp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

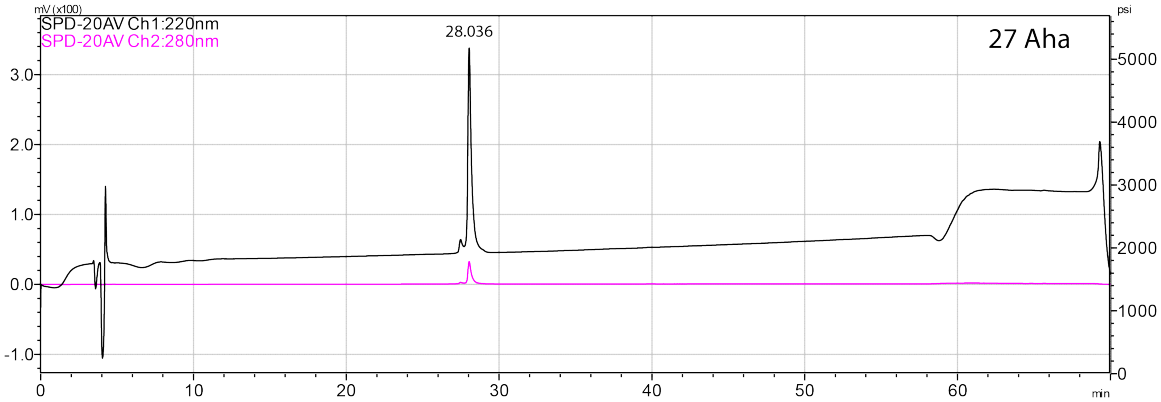


Figure S67. Analytical HPLC Data for WW variant **27X**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

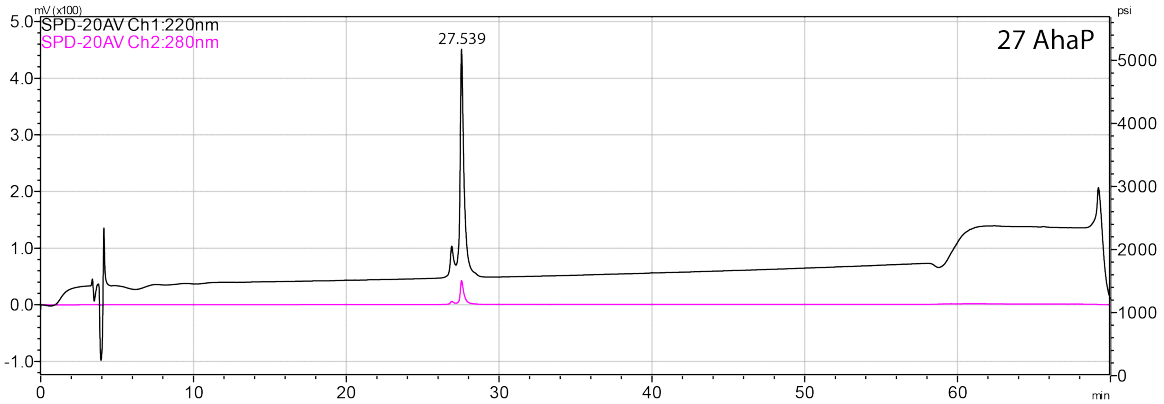


Figure S69. Analytical HPLC Data for WW variant **27Xp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

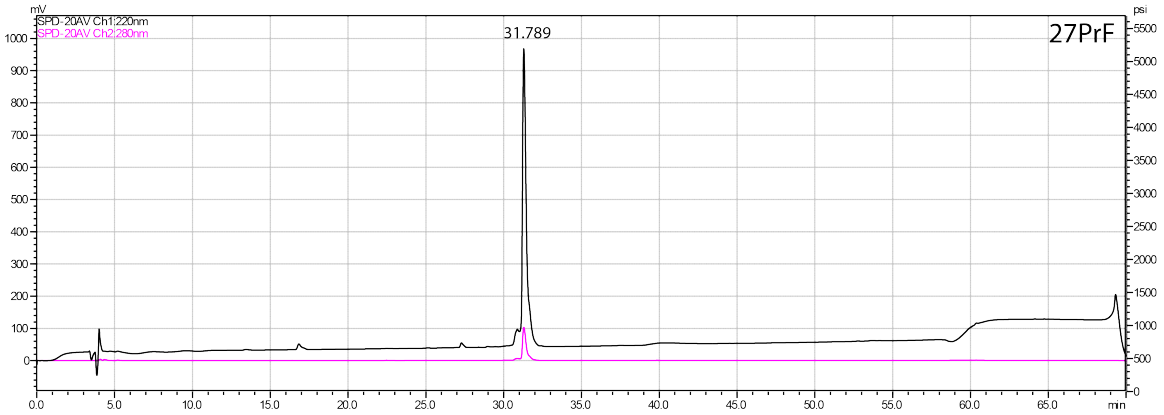


Figure S70. Analytical HPLC Data for WW variant **27Z**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

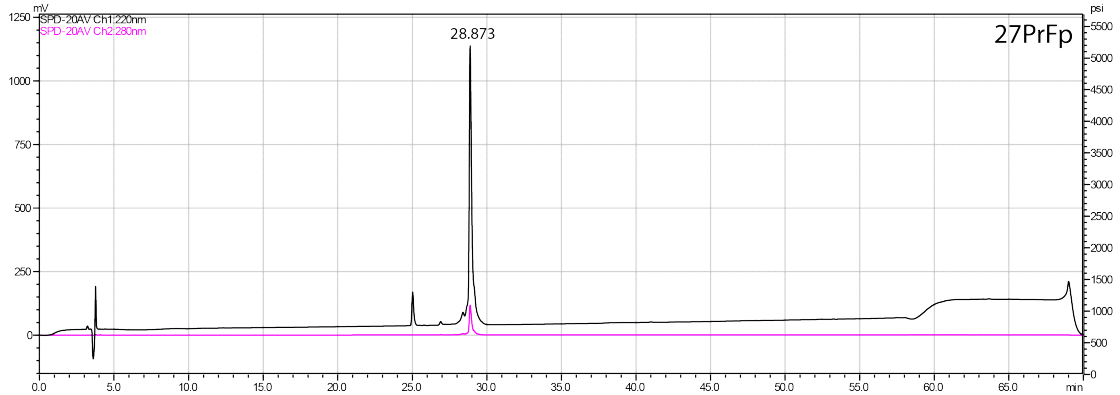


Figure S71. Analytical HPLC Data for WW variant **27Zp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

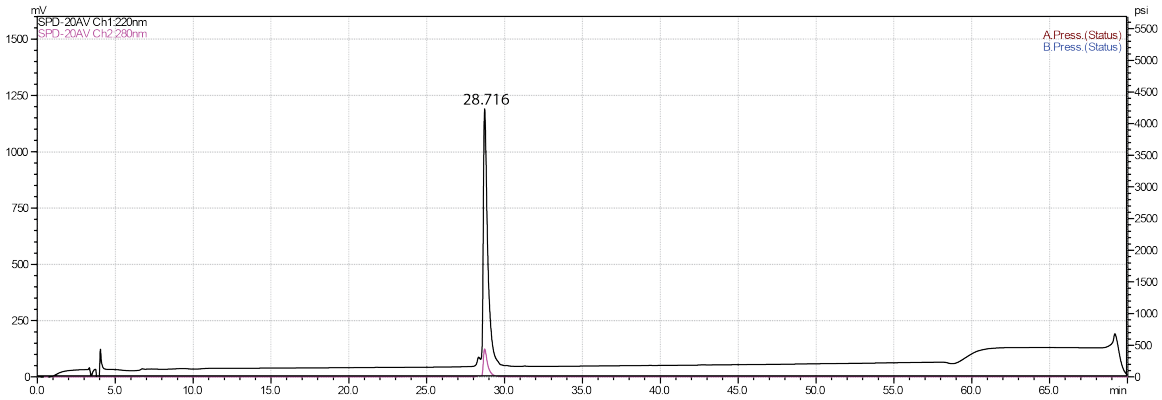


Figure S72. Analytical HPLC Data for WW variant **27Nbp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

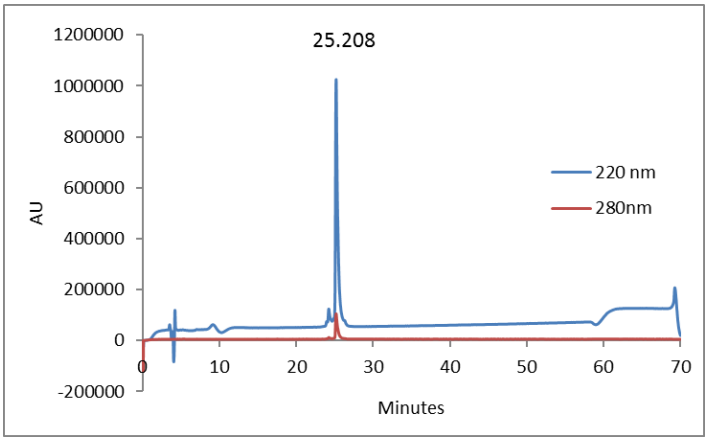


Figure S73. Analytical HPLC Data for WW variant **29Q**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

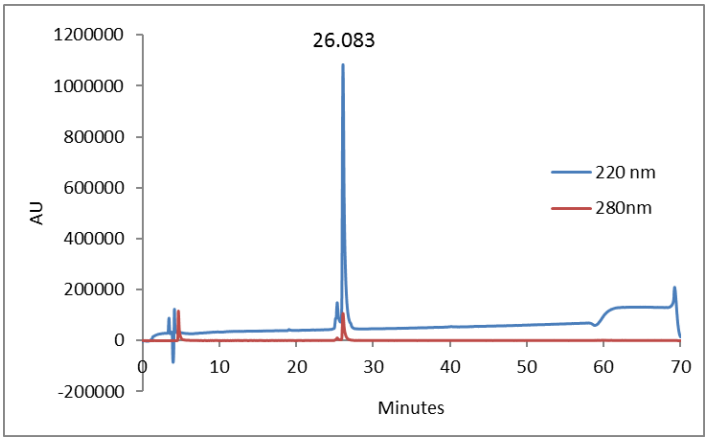


Figure S74. Analytical HPLC Data for WW variant **29Qp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

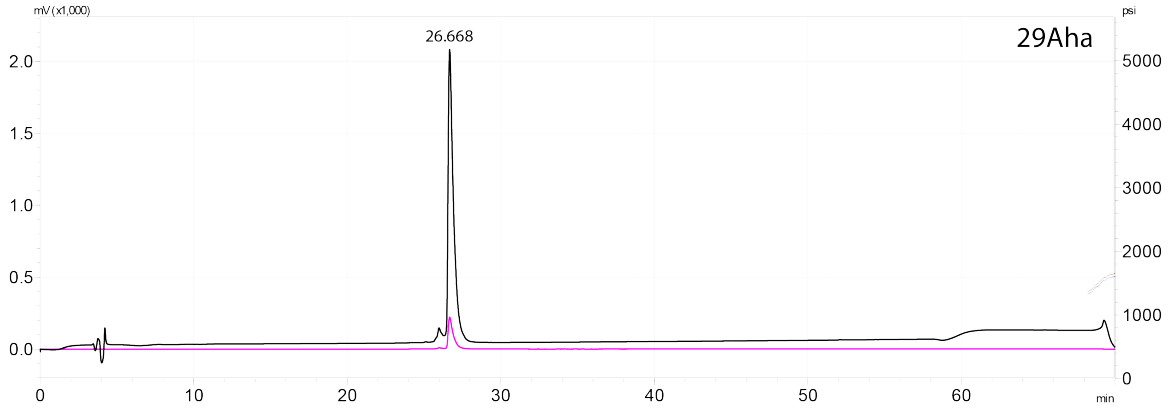


Figure S75. Analytical HPLC Data for WW variant **29X**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

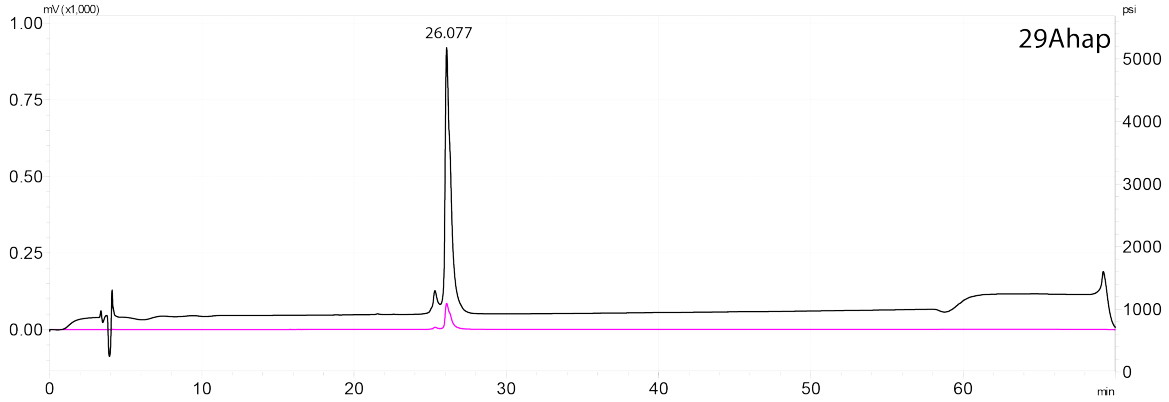


Figure S76. Analytical HPLC Data for WW variant **29Xp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

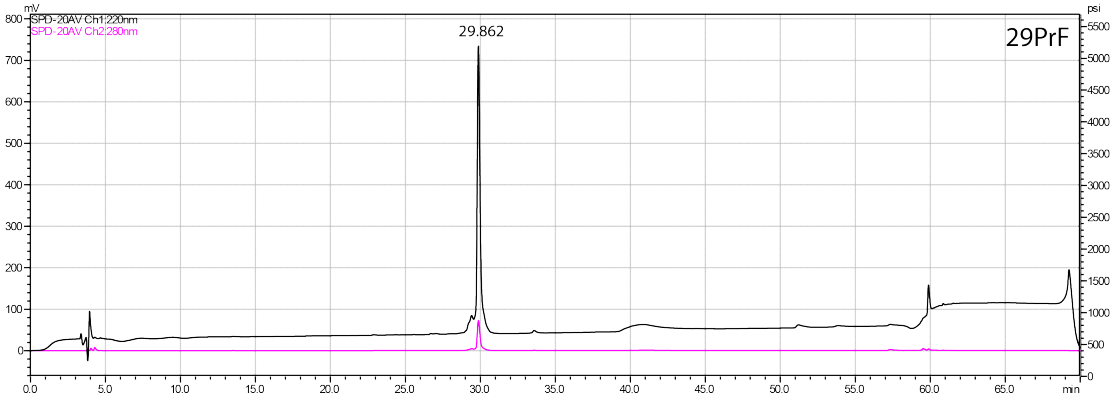


Figure S77. Analytical HPLC Data for WW variant **29Z**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

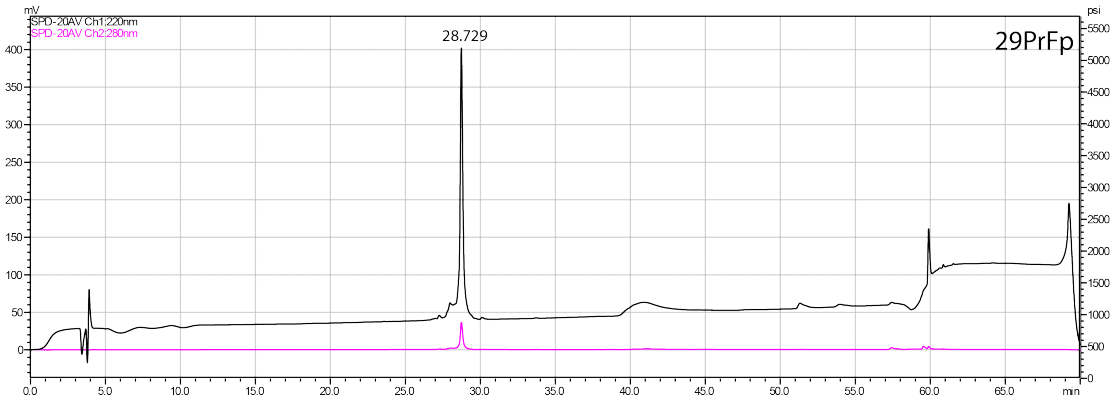


Figure S78. Analytical HPLC Data for WW variant **29Zp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

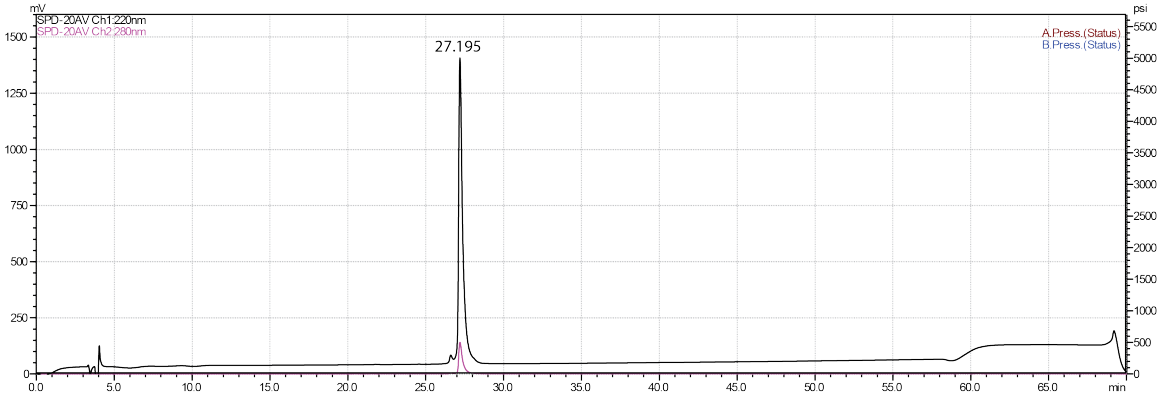


Figure S79. Analytical HPLC Data for WW variant **29Nbp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

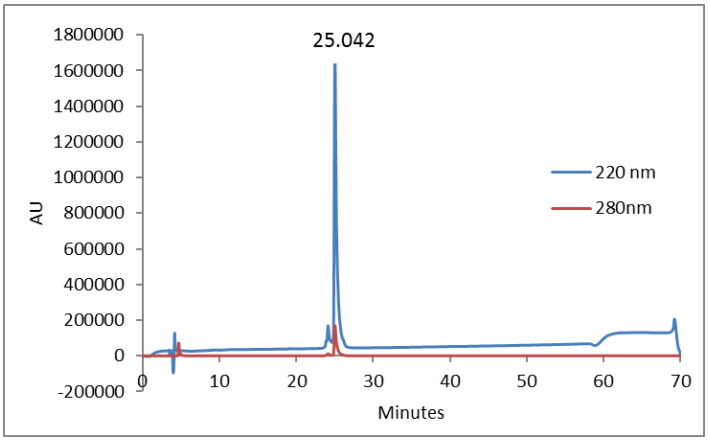


Figure S80. Analytical HPLC Data for WW variant **32Q**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

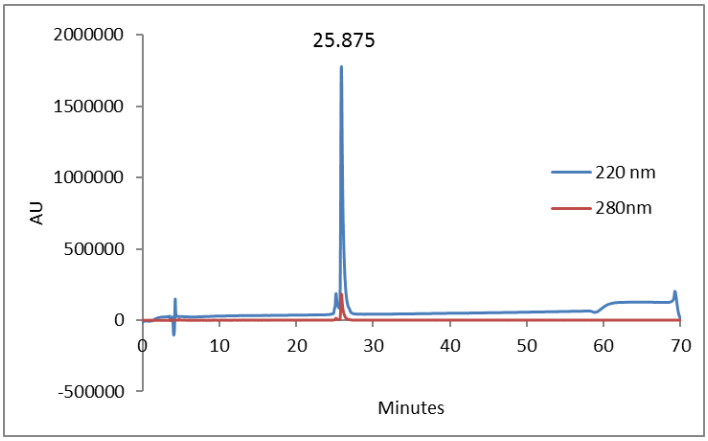


Figure S81. Analytical HPLC Data for WW variant **32Qp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

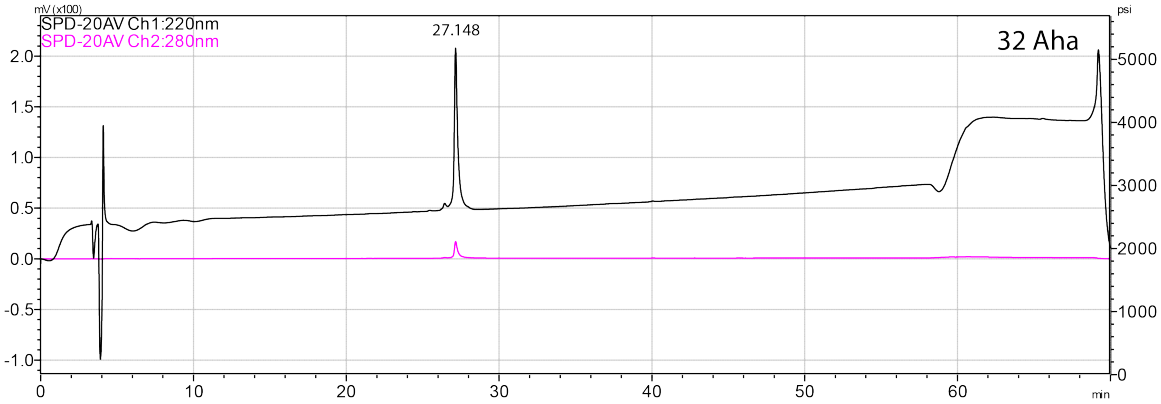


Figure S82. Analytical HPLC Data for WW variant **32X**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

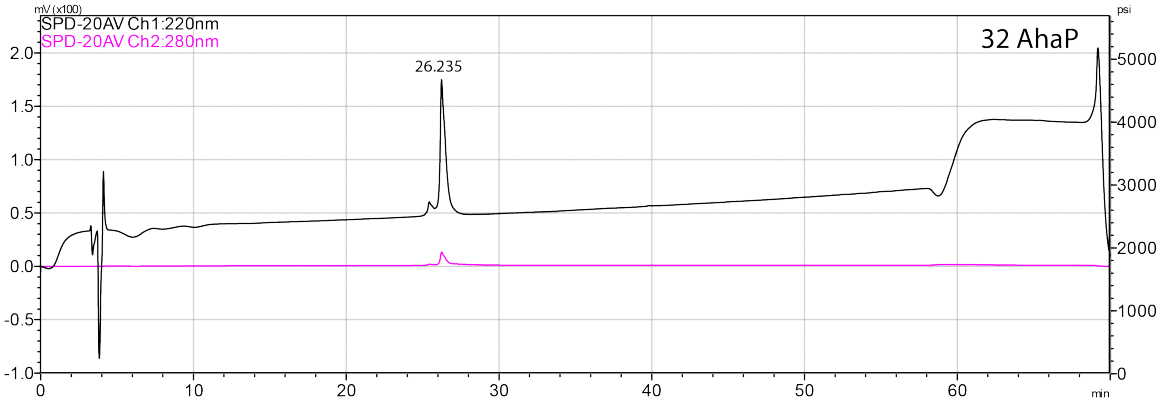


Figure S83. Analytical HPLC Data for WW variant **32Xp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

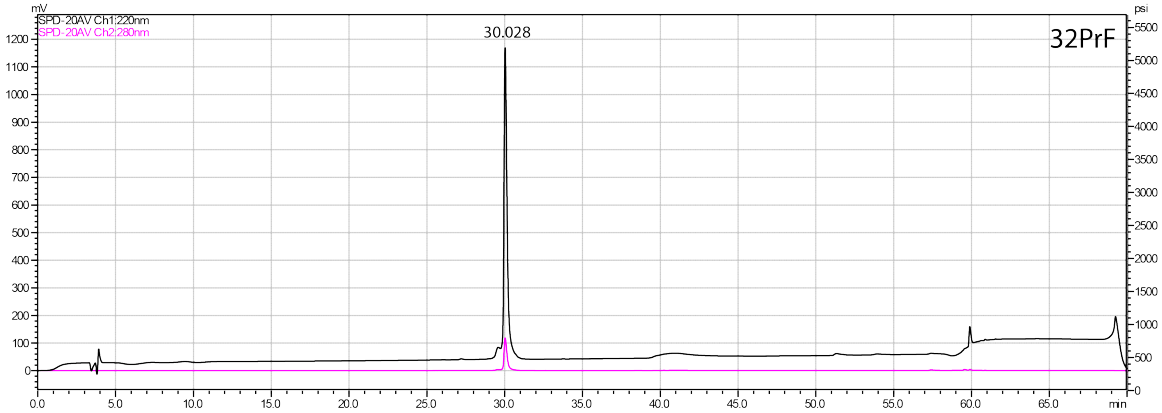


Figure S84. Analytical HPLC Data for WW variant **32Z**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

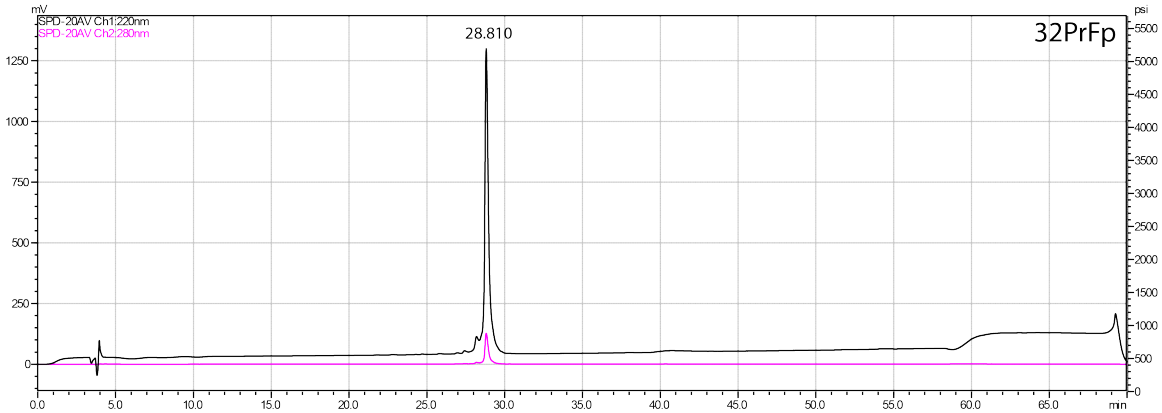


Figure S85. Analytical HPLC Data for WW variant **32Zp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

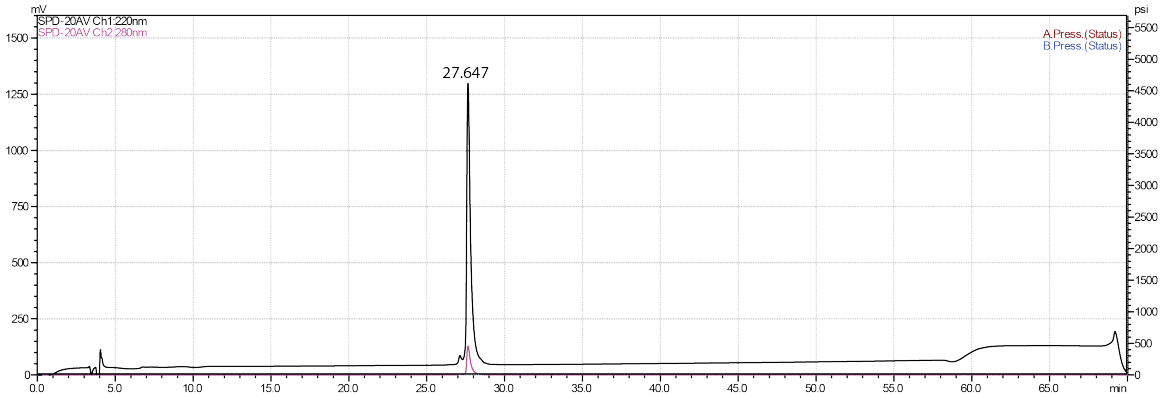


Figure S86. Analytical HPLC Data for WW variant **32Nbp**. Protein solution was injected onto a C18 analytical column and eluted using a linear gradient of 10-60% B (A=H2O, 0.1% TFA; B= MeCN, 0.1% TFA) over 50 minutes, followed by a 10 minute rinse (95% B), and a 10 minute column re-equilibration (10% B) with a flow rate of 1 mL/min.

2. Biophysical Characterization of WW Variants

Measurements were made with an Aviv 420 Circular Dichroism Spectropolarimeter, using quartz cuvettes with a path length of 0.1 cm. Protein solutions were prepared in 20 mM sodium phosphate buffer, pH 7, and protein concentrations were determined spectroscopically based on tyrosine and tryptophan absorbance at 280 nm in 6 M guanidine hydrochloride + 20 mM sodium phosphate (ε_{Trp} = 5690 $M^{-1}cm^{-1}$, ε_{Tyr} = 1280 $M^{-1}cm^{-1}$).[5] CD spectra of 30 μM solutions were obtained from 260 to 200 nm at 25°C. Variable temperature CD data were obtained at least in triplicate by monitoring the molar ellipticity at 222 nm of 30 μM solutions each protein variant (30 μM) in 20 mM sodium phosphate (pH 7) from 1 to 95°C at 2 °C intervals, with 120 s equilibration time between data points and 30 s averaging time.

Triplicate variable temperature CD data for each WW variant and their individual variants were fit globally to a two-state model for thermally-induced unfolding of a monomeric proteins as shown in equations S1–S3:

$$[\theta] = \frac{(D_0 + D_1 \cdot T) + K_f(N_0 + N_1 \cdot T)}{1 + K_f}, \tag{S1}$$

where [θ] is molar ellipticity; T is temperature in Kelvin; D_0 is the *y*-intercept and D_1 is the slope of the post-transition baseline; N_0 is the *y*-intercept and N_1 is the slope of the pre-transition baseline; and K_f is the temperature-dependent folding equilibrium constant. K_f is related to the temperature-dependent free energy of folding $\Delta G_f(T)$ according to the following equation:

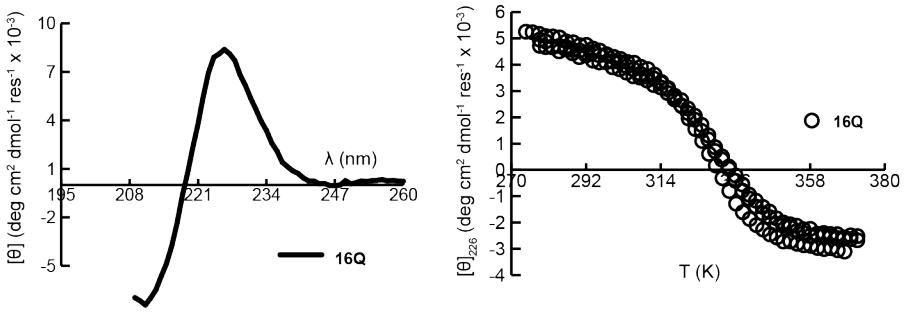
$$K_f = \exp\left[\frac{-\Delta G_f(T)}{RT}\right], \tag{S2}$$

where R is the universal gas constant (0.0019872 kcal/mol/K). $\Delta G_f(T)$ was fit to the following equation:

$$\Delta G_f = \frac{\Delta H(T_m)\cdot(T_m - T)}{T_m} + \Delta C_p \cdot (T - T_m - T \cdot \ln\left[\frac{T}{T_m}\right]) \tag{S3}$$

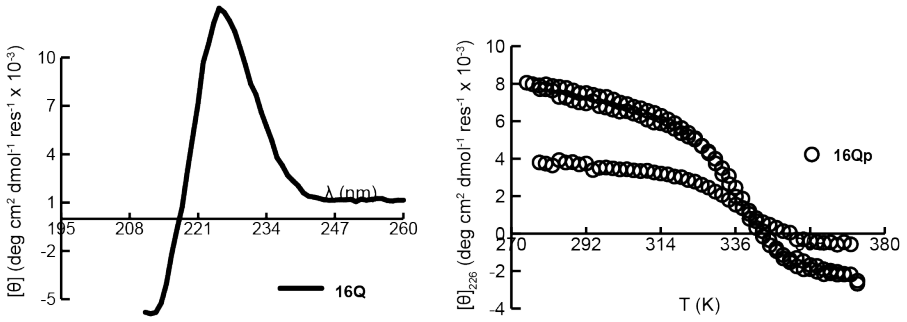
where the fit parameters are T_m (the midpoint of the unfolding transition; the temperature at which $\Delta G_f = 0$); $\Delta H(T_m)$, the change in enthalpy upon folding at T_m; and ΔC_p, the change in heat capacity upon folding. The parameters for equations S1-S3 were used to calculate the values of the folding free energy ΔG_f for WW variants

in the main text. Far-UV CD spectra and variable temperature CD data for these compounds are shown below in Figures S87-S116.



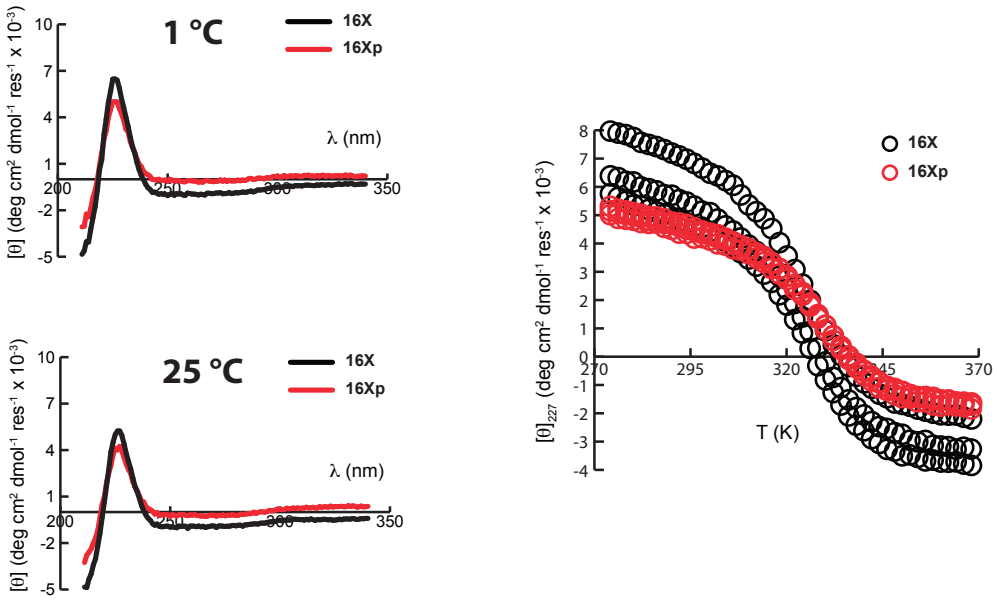
Protien	Tm	ΔH(Tm)	R^2	rmsd error
16Q	329.8 ± 0.1	-28.7 ± 0.3	0.9997	0.0497

Figure S87. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **16Q** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



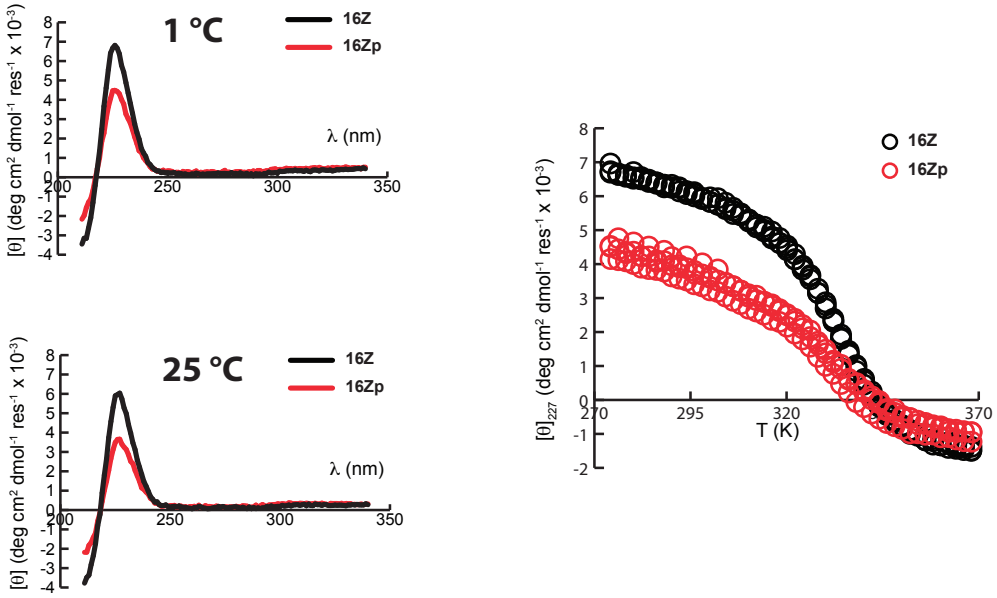
Protien	Tm	ΔH(Tm)	R^2	rmsd error
16Qp	336.6 ± 0.1	-32.8 ± 0.6	0.9994	0.0859

Figure S88. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **16Qp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



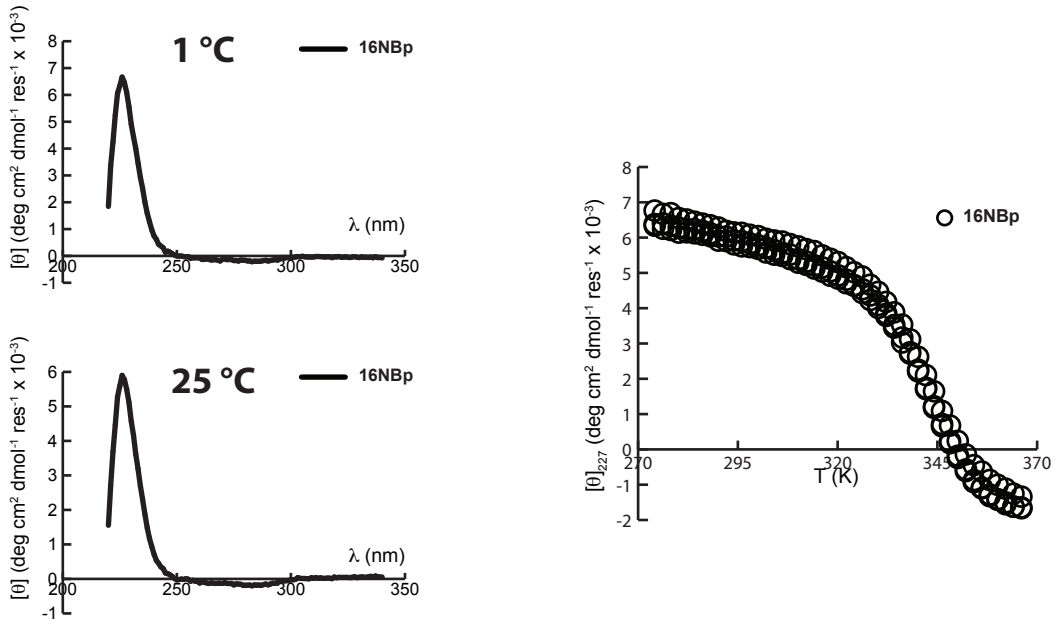
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
16X	325.9 ± 0.3	-29.0 ± 0.5	-0.52 ± 0.10	0.9999	0.048
16Xp	330.0 ± 0.3	-31.8 ± 0.4	-0.36 ± 0.12	0.9999	0.030

Figure S89. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variants **16X** (black) and **16Xp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



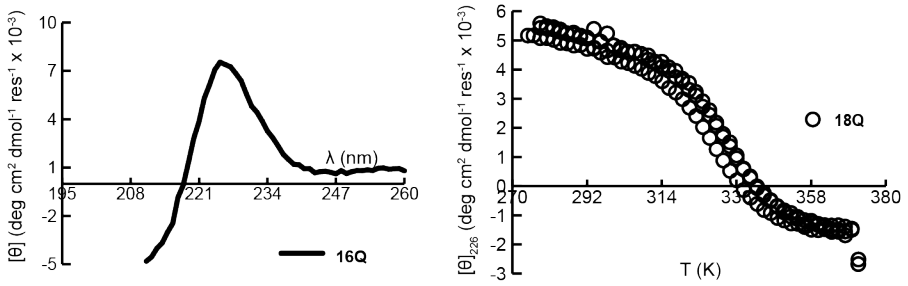
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
16Z	333.4 ± 0.2	-31.8 ± 0.6	-0.69 ± 0.04	0.9998	0.045
16Zp	332.8 ± 0.5	-33.2 ± 2.1	-0.82 ± 0.08	0.9992	0.072

Figure S90. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variants **16Z** (black) and **16Zp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



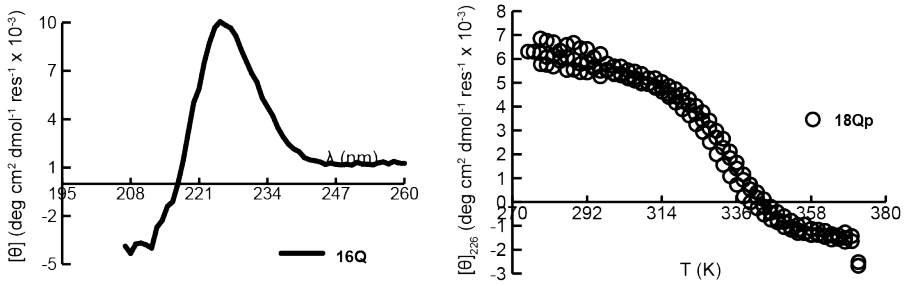
Peptide	T_m	$\Delta H(T_m)$	ΔC_p	R^2	rmsd error
16NBp	343.40 ± 0.05	-36.13 ± 0.27	-0.64 ± 0.03	0.99989	0.02993

Figure S91. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **16NBp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



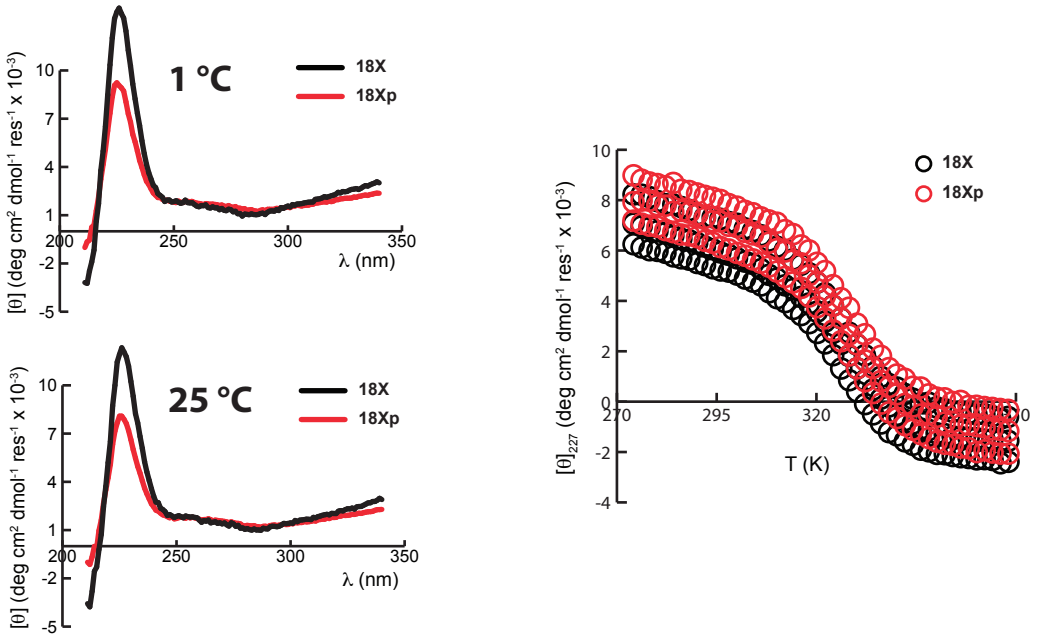
Protien	Tm	ΔH(Tm)	R^2	rmsd error
18Q	331.6 ± 0.2	-28.8 ± 0.5	0.9995	0.0660

Figure S92. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **18Q** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



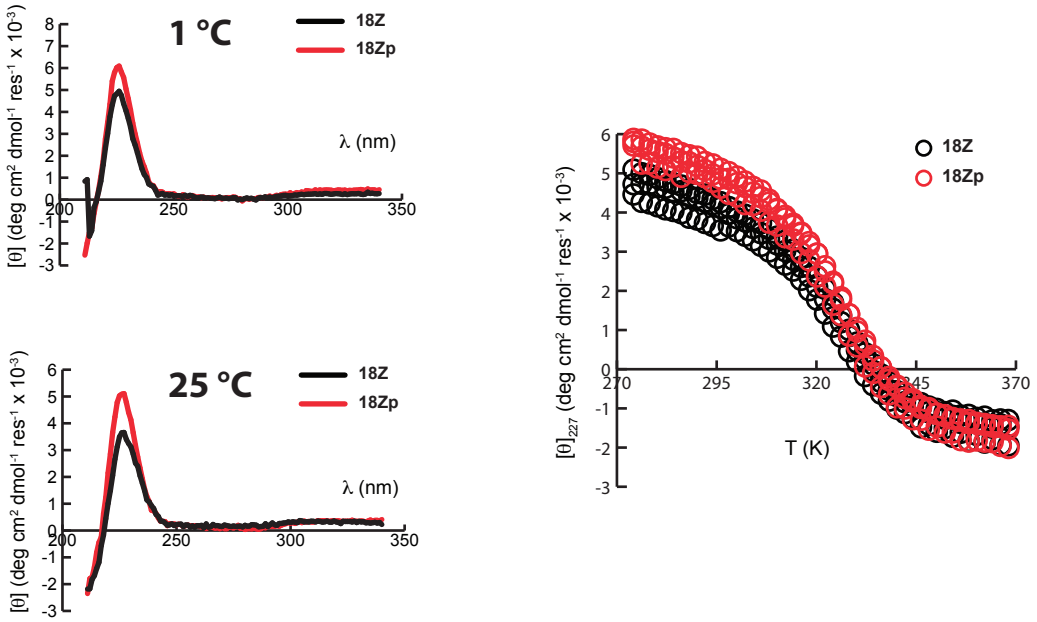
Protien	Tm	ΔH(Tm)	R^2	rmsd error
18Qp	332.9 ± 0.2	-30.6 ± 0.6	0.9994	0.0825

Figure S93. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variant **18Qp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



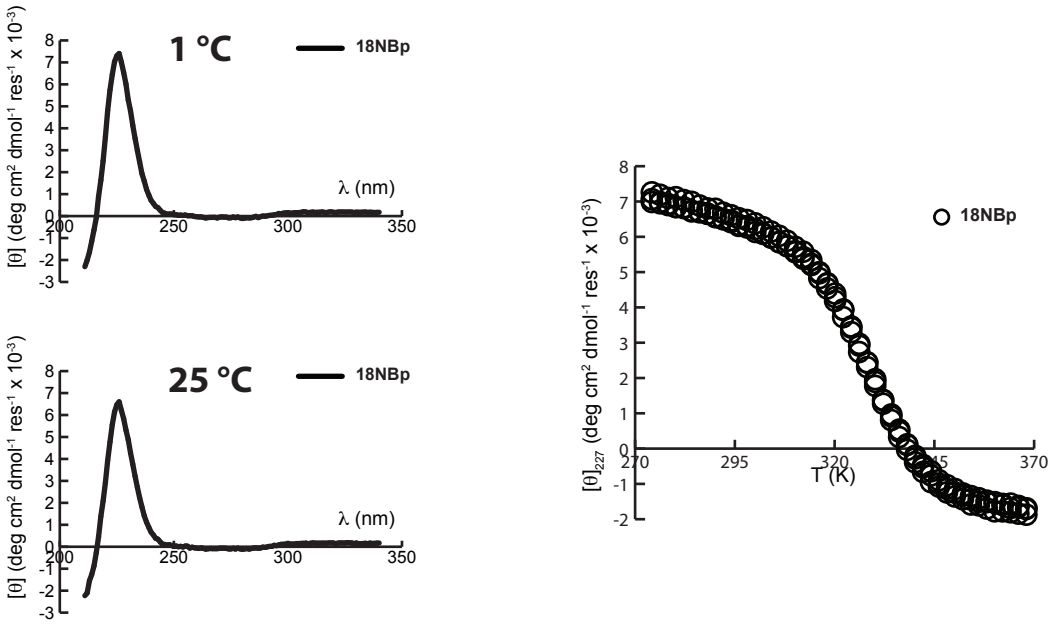
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
18X	327.6 ± 0.4	-31.5 ± 0.6	-0.48 ± 0.15	0.9998	0.045
18Xp	329.2 ± 0.2	-30.9 ± 0.5		0.9998	0.053

Figure S94. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **18X** (black) and **18Xp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



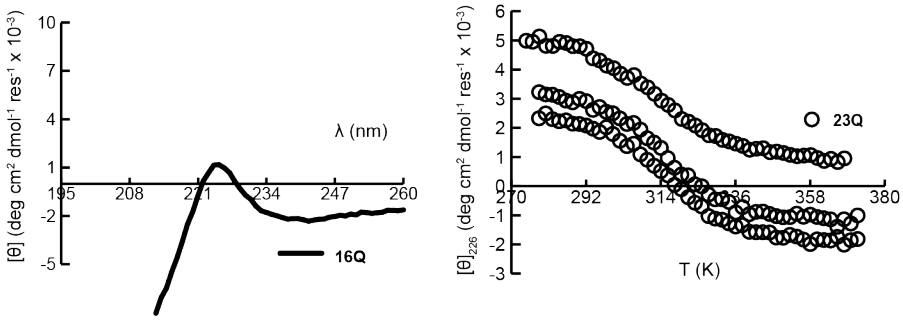
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
18Z	326.0 ± 0.2	-28.3 ± 0.7	-0.63 ± 0.07	0.9997	0.046
18Zp	326.6 ± 0.4	-27.7 ± 0.7	-0.53 ± 0.12	0.9996	0.058

Figure S95. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **18Z** (black) and **18Zp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



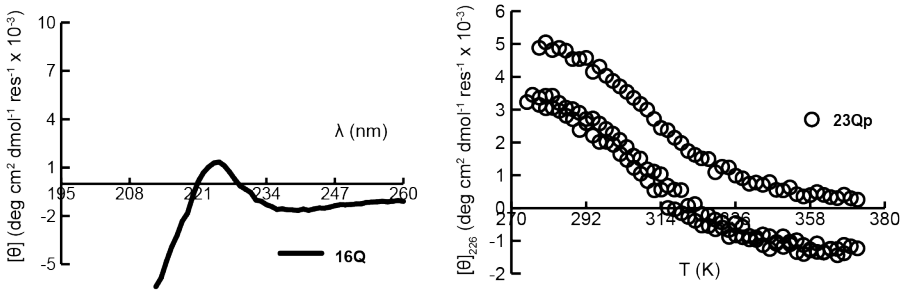
Peptide	T_m	$\Delta H(T_m)$	ΔC_p	R^2	rmsd error
18NBp	330.44 ± 0.11	-29.05 ± 0.21	0.09 ± 0.05	0.99987	0.04050

Figure S96. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variant **18NBp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



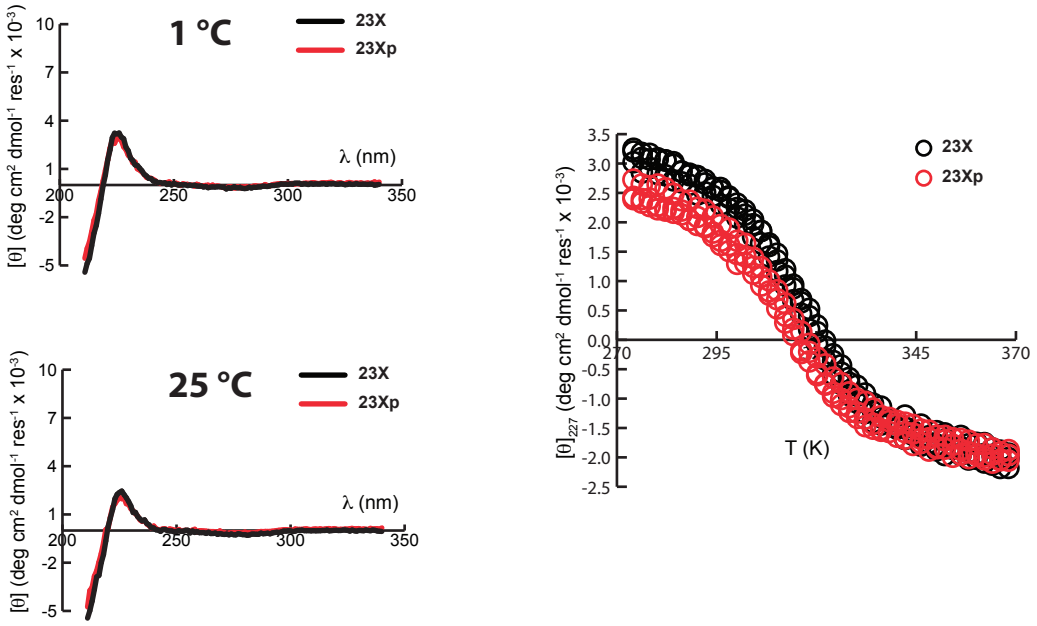
Protien	Tm	ΔH(Tm)	R^2	rmsd error
23Q	312.6 ± 0.2	-19.2 ± 0.4	0.9991	0.0946

Figure S97. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **23Q** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



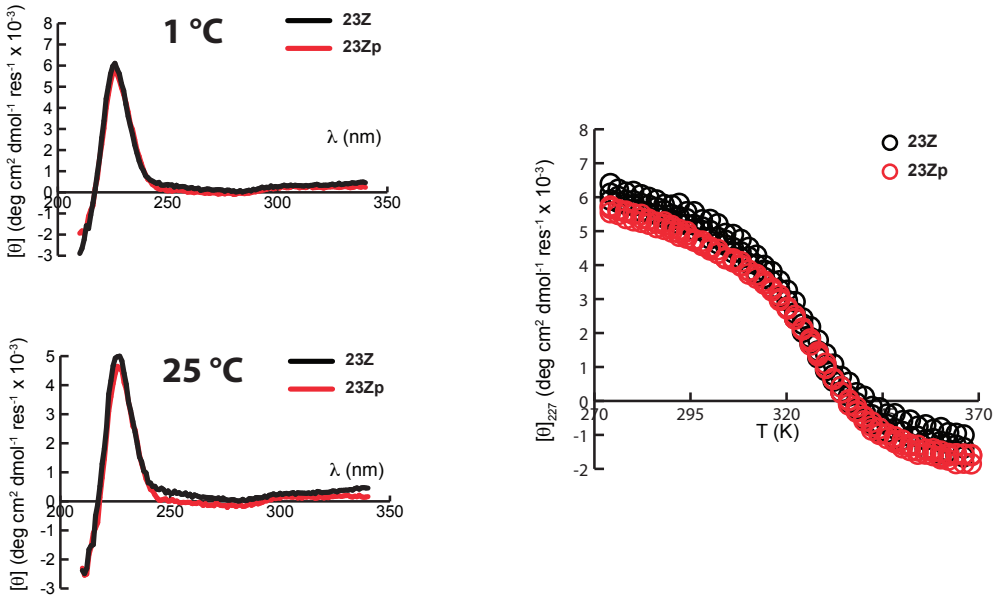
Protien	Tm	ΔH(Tm)	R^2	rmsd error
23Qp	307.7 ± 0.3	-14.7 ± 0.3	0.9992	0.0912

Figure S98. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **23Qp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



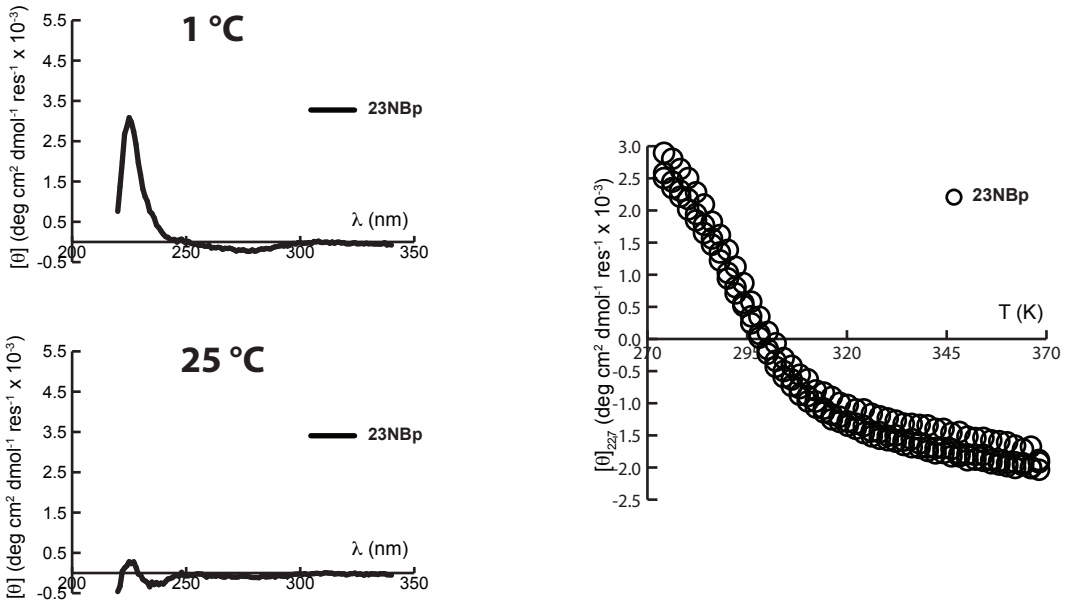
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
23X	317.8 ± 0.3	-25.3 ± 0.8		0.9993	0.052
23Xp	314.9 ± 0.4	-27.2 ± 1.2		0.9988	0.061

Figure S99. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **23X** (black) and **23Xp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



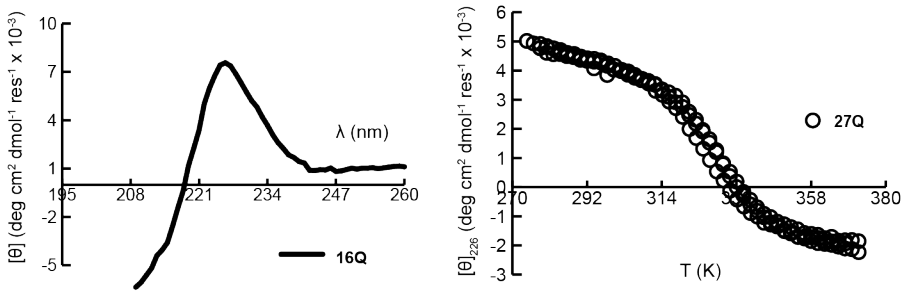
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
23Z	325.2 ± 0.2	-26.6 ± 0.6	-0.59 ± 0.06	0.9998	0.042
23Zp	328.7 ± 0.2	-28.6 ± 0.7	-0.65 ± 0.04	0.9998	0.042

Figure S100. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **23Z** (black) and **23Zp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



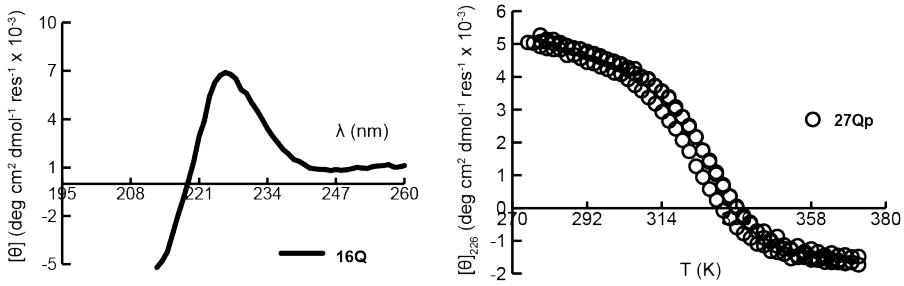
Protein	T_m (K)	$\Delta H(T_m)$ (kcal/mol)	ΔC_p (kcal/mol/K)	R^2	std error
23NBp	298.0 ± 0.3	-20.3 ± 0.5	0.59 ± 0.04	0.9997	0.025

Figure S101. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **23NBp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



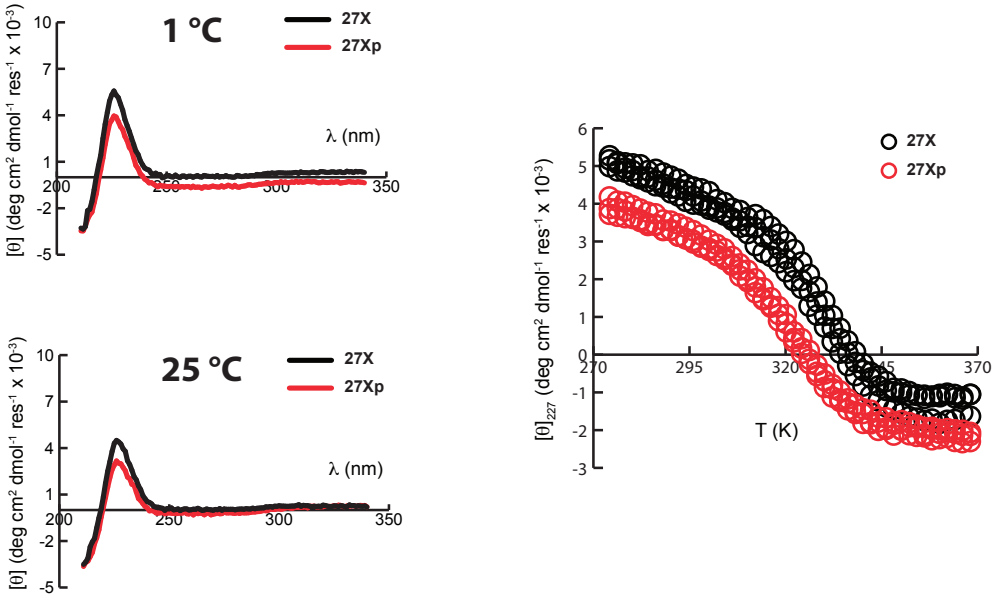
Protien	Tm	ΔH(Tm)	R^2	rmsd error
27Q	329.5 ± 0.2	-27.5 ± 0.4	0.9996	0.0575

Figure S102. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **27Q** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



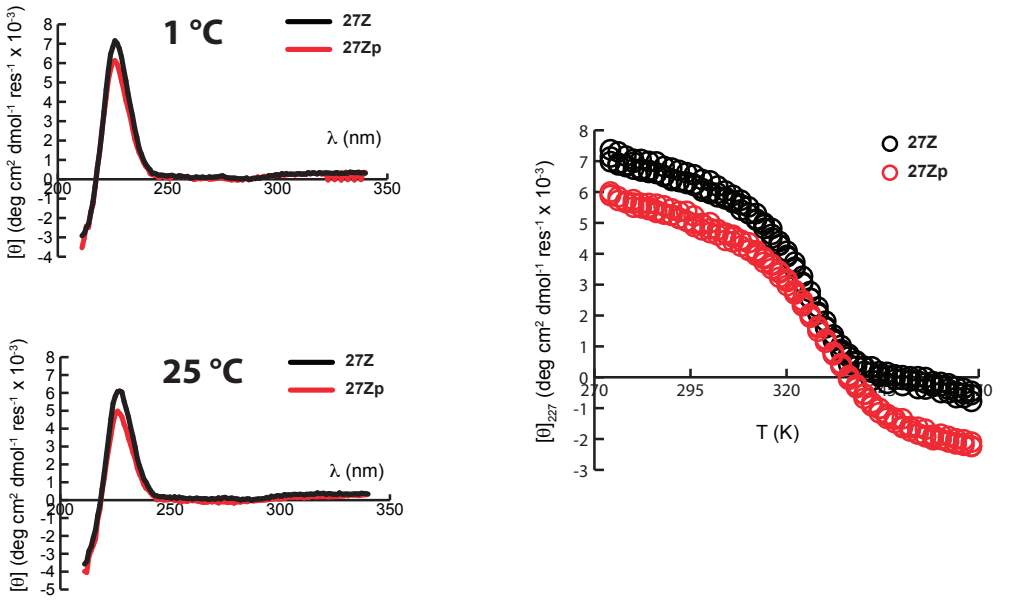
Protien	Tm	ΔH(Tm)	R^2	rmsd error
27Qp	362.3 ± 0.1	-26.9 ± 0.1	0.9998	0.0429

Figure S103. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variant **27Qp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



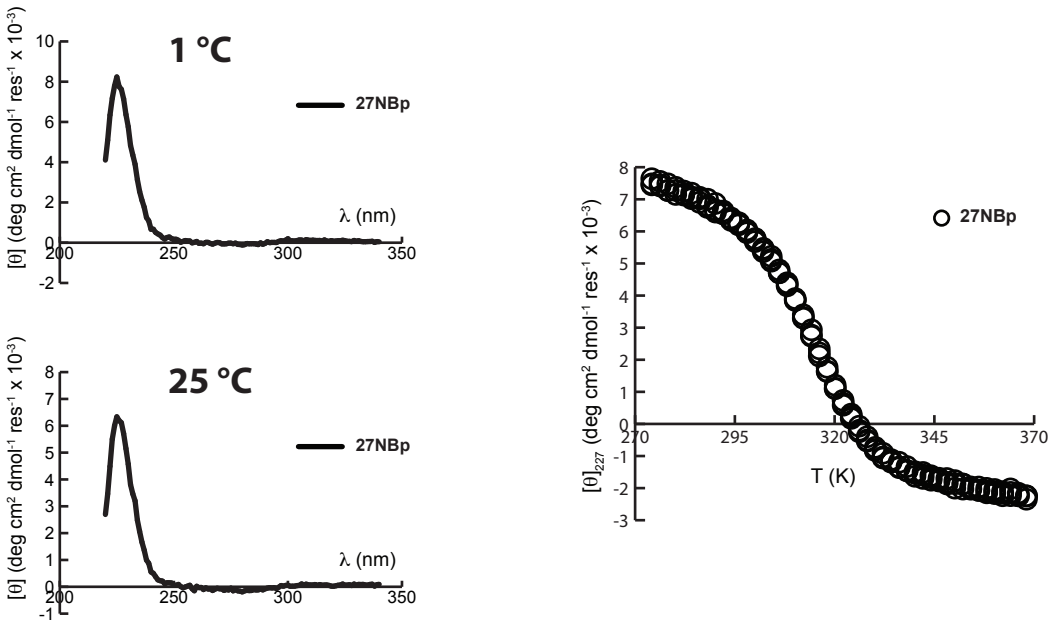
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
27X	331.7 ± 0.2	-32.5 ± 0.8	-0.67 ± 0.07	0.9992	0.071
27Xp	325.3 ± 0.2	-25.6 ± 0.4		0.9994	0.058

Figure S104. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **27X** (black) and **27Xp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



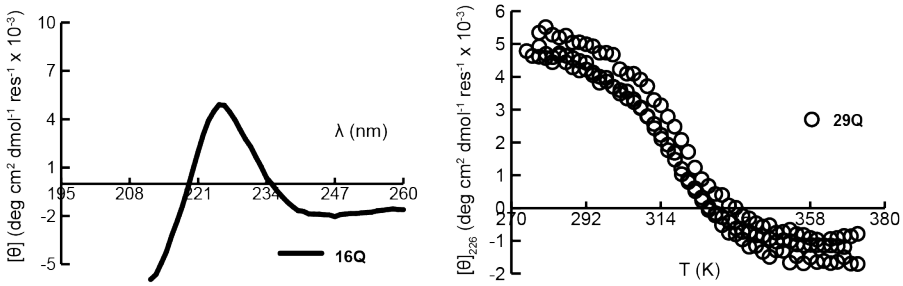
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
27Z	325.1 ± 0.2	-37.5 ± 0.9	-0.98 ± 0.06	0.9997	0.058
27Zp	331.6 ± 0.1	-26.7 ± 0.3	-	0.9997	0.057

Figure S105. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **27Z** (black) and **27Zp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



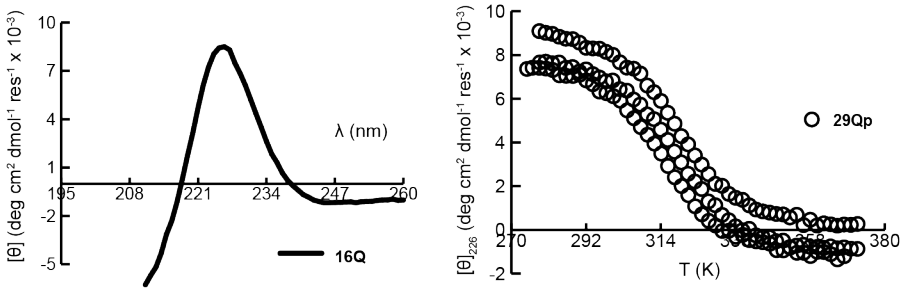
Peptide	T_m	$\Delta H(T_m)$	ΔC_p	R^2	rmsd error
27NBp	314.65 ± 0.35	-26.04 ± 0.66	-0.53 ± 0.08	0.99993	0.03582

Figure S106. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variant **27NBp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



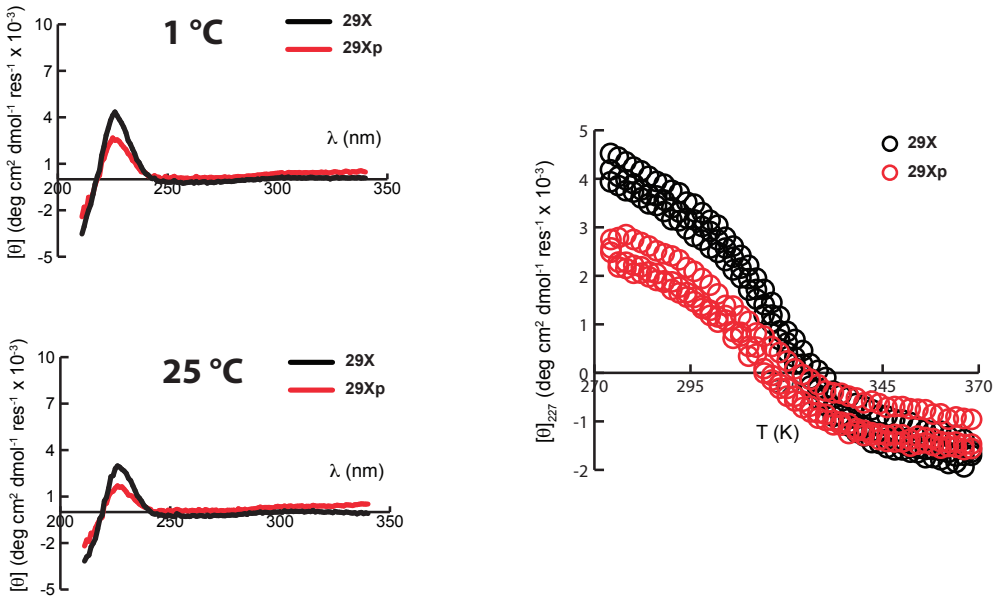
Protien	Tm	ΔH(Tm)	R^2	rmsd error
29Q	318.4 ± 0.5	-23.9 ± 0.7	0.9993	0.0843

Figure S107. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **29Q** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



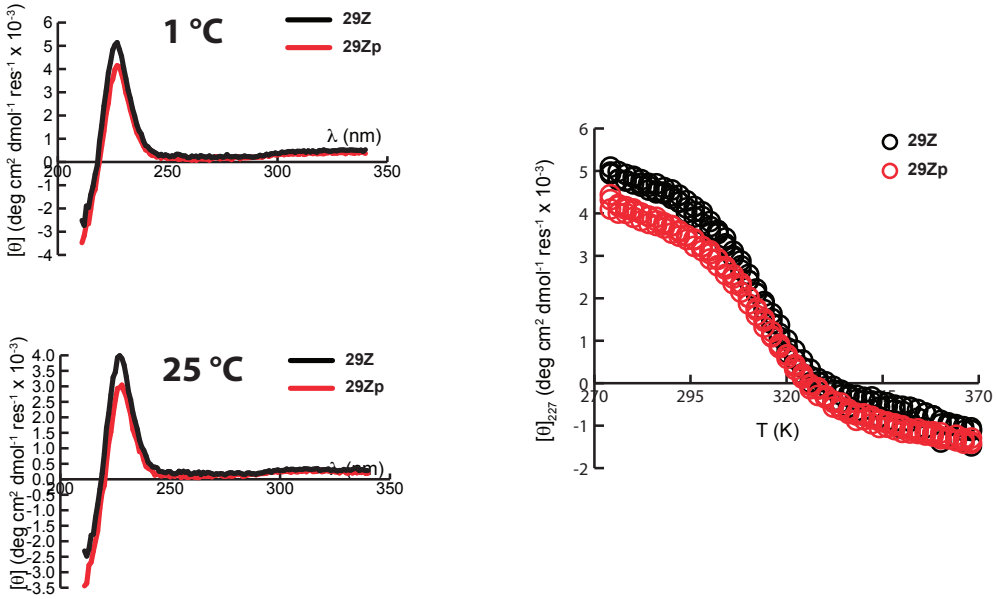
Protien	Tm	ΔH(Tm)	R^2	rmsd error
29Qp	318.4 ± 0.5	-23.9 ± 0.7	0.9997	0.0864

Figure S108. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **29Qp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



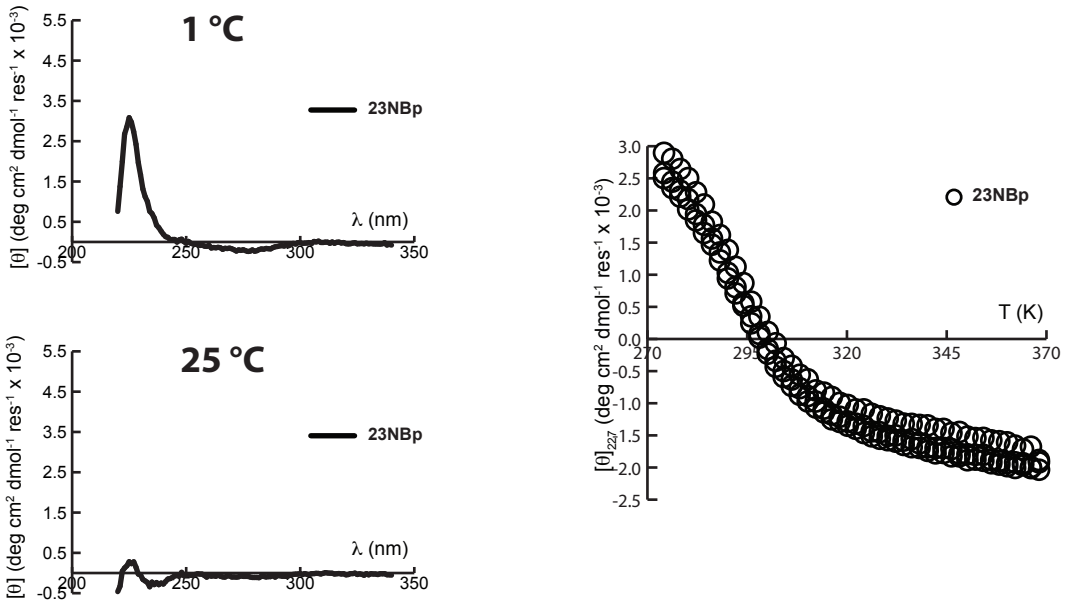
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
29X	320.7 ± 0.1	-26.1 ± 0.3		0.9996	0.046
29Xp	315.7 ± 0.6	-24.8 ± 1.4		0.9986	0.056

Figure S109. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **29X** (black) and **29Xp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



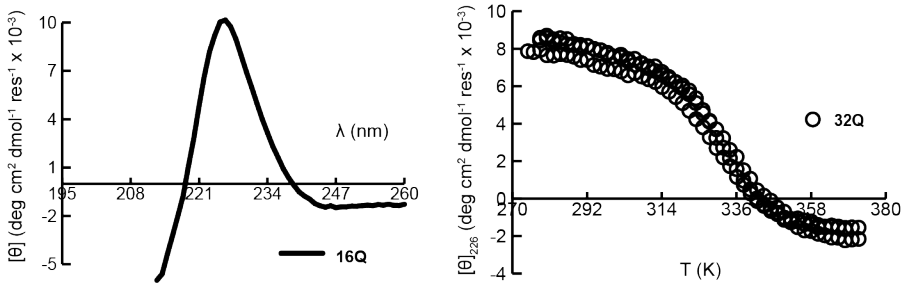
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
29Z	312.5 ± 0.4	-27.8 ± 1.1	-0.92 ± 0.08	0.9996	0.052
29Zp	316.3 ± 0.2	-26.2 ± 0.5	-	0.9998	0.033

Figure S110. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **29Z** (black) and **29Zp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



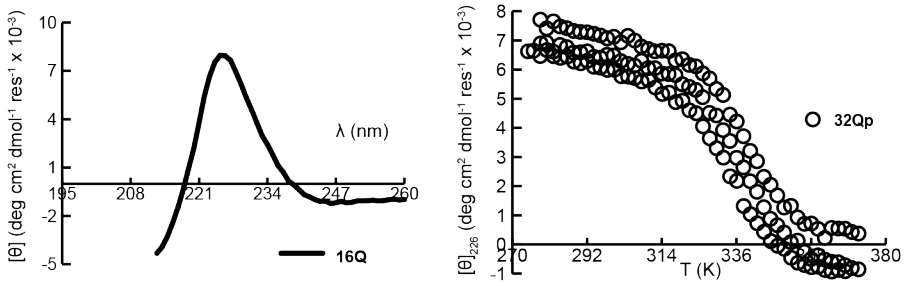
Protein	T_m (K)	$\Delta H(T_m)$ (kcal/mol)	ΔC_p (kcal/mol/K)	R^2	std error
23NBp	298.0 ± 0.3	-20.3 ± 0.5	0.59 ± 0.04	0.9997	0.025

Figure S111. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **29NBp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



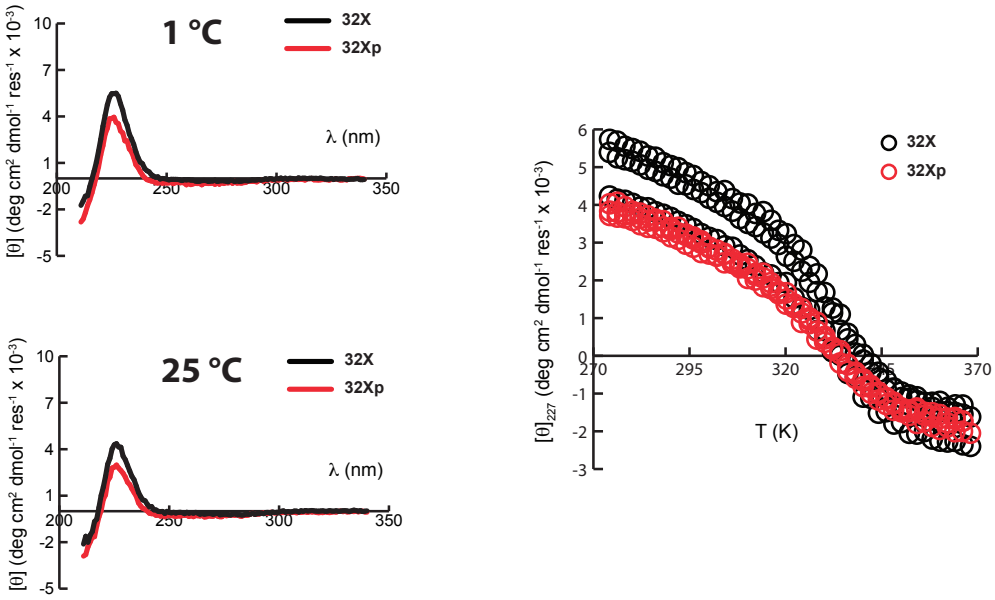
Protien	Tm	ΔH(Tm)	R^2	rmsd error
32Q	331.5 ± 0.2	-28.8 ± 0.5	0.9996	0.09997

Figure S112. CD spectra (50 μM) and variable temperature CD data (50 μM) for WW variant **32Q** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



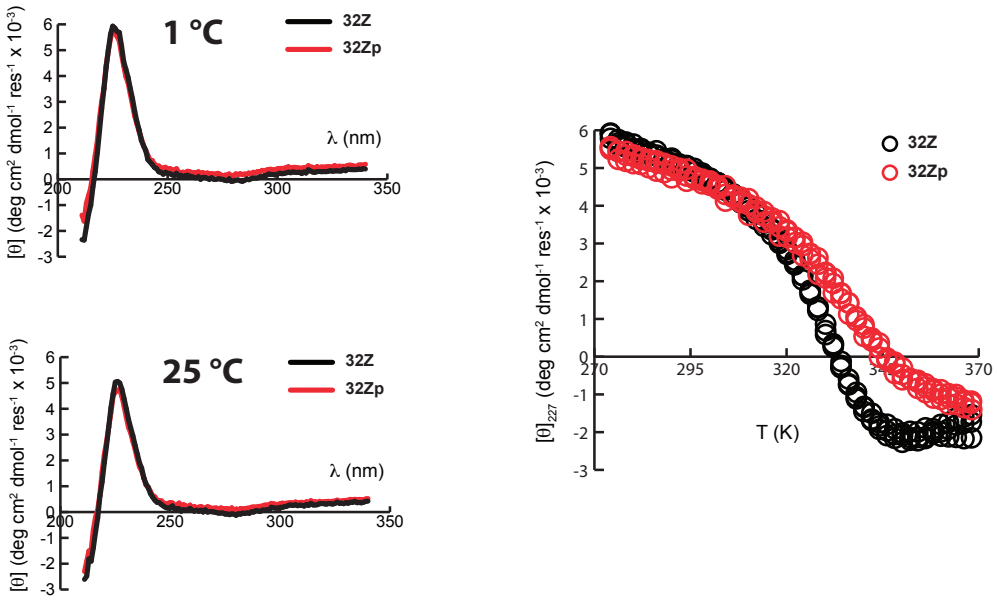
Protien	Tm	ΔH(Tm)	R^2	rmsd error
32Qp	336.1 ± 0.2	-32.4 ± 0.7	0.9996	0.09554

Figure S113. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variant **32Qp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



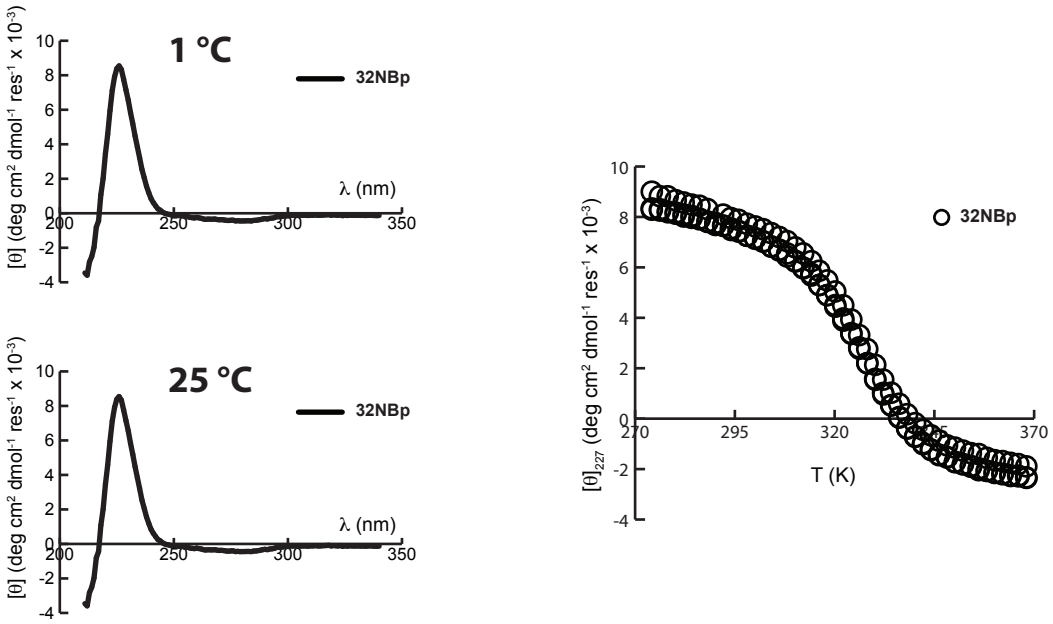
Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
32X	333.4 ± 0.3	-31.9 ± 1.3	-0.69 ± 0.07	0.9995	0.063
32Xp	333.0 ± 0.5	-28.0 ± 1.7	-0.66 0.07	0.9995	0.057

Figure S114. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **32X** (black) and **32Xp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



Peptide	Tm/ K	$\Delta H(T_m)$ / kcal mol^{-1}	ΔC_p / kcal mol^{-1} K^{-1}	R^2	rmsd error
32Z	331.9 ± 0.2	-31.5 ± 0.8	-0.66 ± 0.05	0.9996	0.070
32Zp	336.6 ± 0.3	-23.5 ± 0.6	-0.41 ± 0.05	0.9993	0.062

Figure S115. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variants **32Z** (black) and **32Zp** (red) in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.



Peptide	T_m	$\Delta H(T_m)$	ΔC_p	R^2	rmsd error
32NBp	327.37 ± 0.39	-30.35 ± 0.42	-0.32 ± 0.12	0.99988	0.05042

Figure S116. CD spectra (50 µM) and variable temperature CD data (50 µM) for WW variant **32NBp** in 20 mM sodium phosphate, pH 7. Fit parameters appear in the table, along with standard errors.

3. Proteolysis Experiments

50 μM solutions of PEGylated WW variants or their non-PEGylated counterparts in 20 mM sodium phosphate buffer (pH 7) were incubated at ambient temperature with 17 μg/mL proteinase K for up to 90 minutes. At regular time points, a 50 μL aliquot of the reaction mixture was quenched by addition of 150 μL of a 1% solution (v/v) of trifluoroacetic acid in water. Each quenched aliquot was then analyzed in triplicate by analytical reverse-phase HPLC, using a UV-Vis detector at 220 nm. The amount of each full-length WW variant remaining in solution at each time point was assessed by integrated peak area, expressed as the remaining percentage of the integrated peak area from a control solution that contained no protease. The apparent proteolysis rate constant k (in units of s^{-1}) for each WW variant was calculated by fitting the % area vs. time data to an equation for monoexponential decay by least-squares regression:

$$\text{Area}(t) = A_0 \exp [-k\, t],$$

where t is time (in units of s), A_0 is a constant corresponding to % area at t = 0 s. The apparent proteolysis rate constant k for each variant is inversely related to half-life ($t_{1/2}$, in units of s) according to the following relationship: $t_{1/2} = (\ln 2)/k$; smaller values of k are correlated with longer half-lives. Plots of % area vs. time data and the resulting fits for the WW variants explored here are shown in Figures S117– S178. Apparent rate constants for each variant are shown in the main text. Proteolysis experiments for linear Asn-PEGylated variants and their non-PEGylated counterparts in the presence of proteinase K were reported previously[1], but were performed again here to account for the increased proteinase K concentration employed here relative to what we used previously.

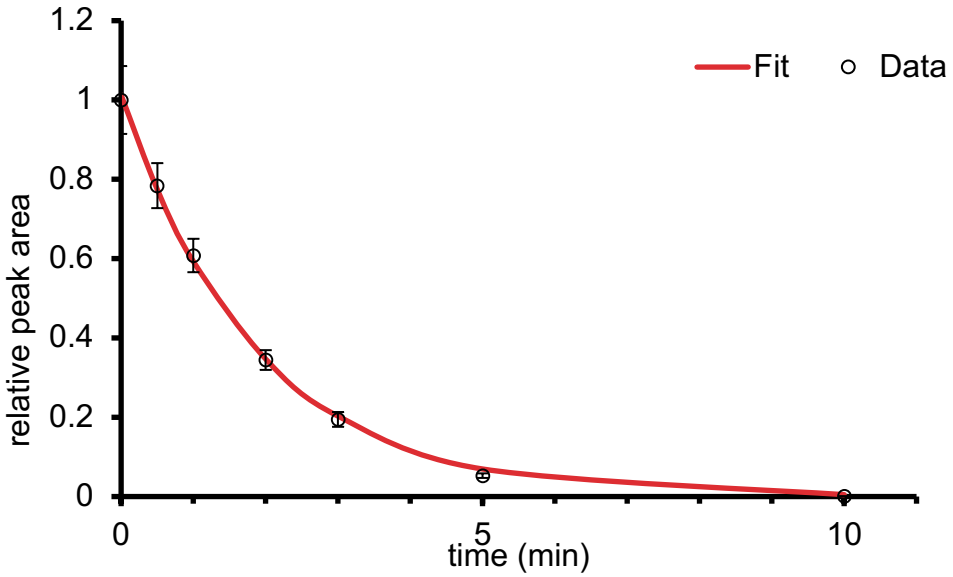


Figure S117. **($t_{1/2}$ = 1.29 ± 0.03 min)** Proteolysis of **16Q** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

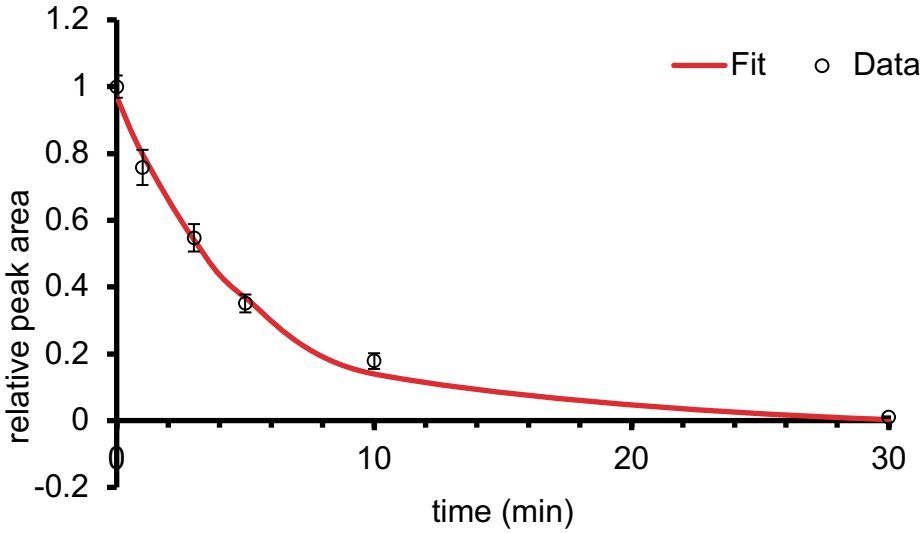


Figure S118. **($t_{1/2}$ = 3.58 ± 0.22 min)** Proteolysis of **16Qp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

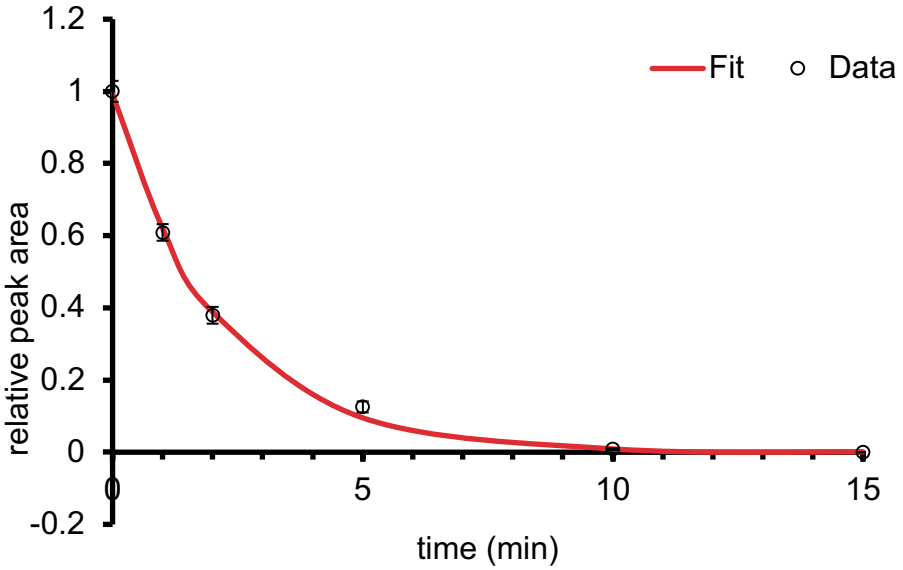


Figure S119. **($t_{1/2}$ = 1.48 ± 0.08 min)** Proteolysis of **18Q** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

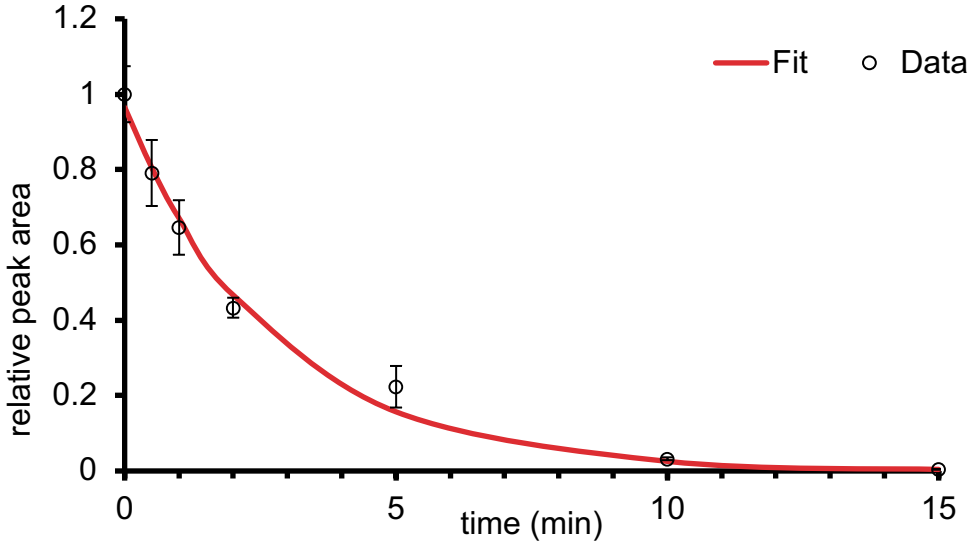


Figure S120. **($t_{1/2}$ = 1.91 ± 0.17 min)** Proteolysis of **18Qp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

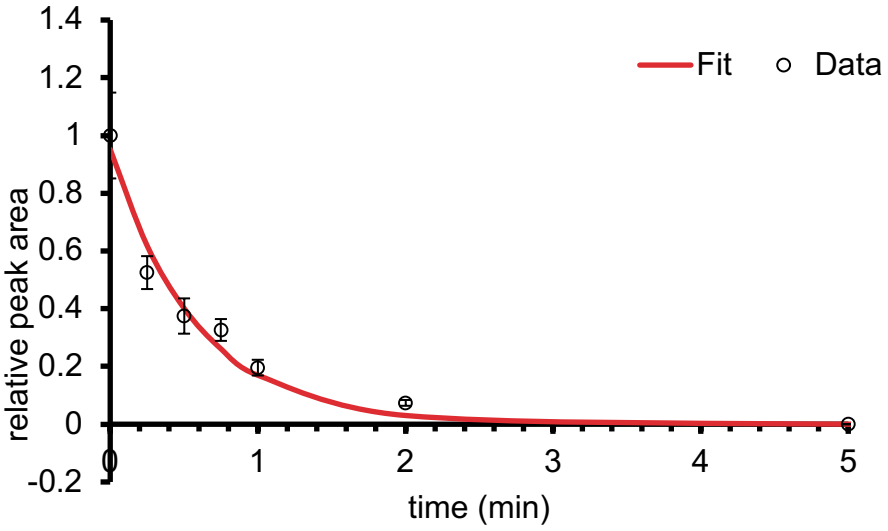


Figure S121. **($t_{1/2}$ = 0.40 ± 0.04 min)** Proteolysis of **19Q** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

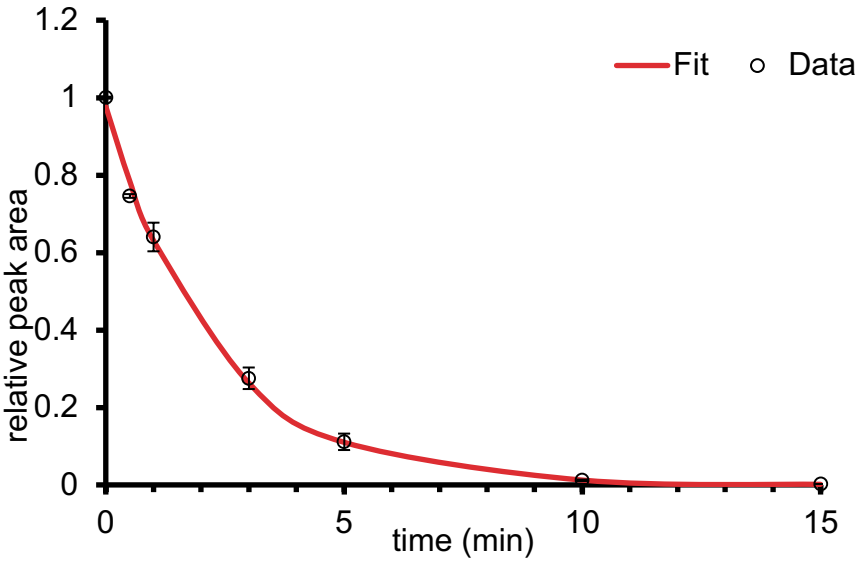


Figure S122. **($t_{1/2}$ = 1.59 ± 0.08 min)** Proteolysis of **19Qp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

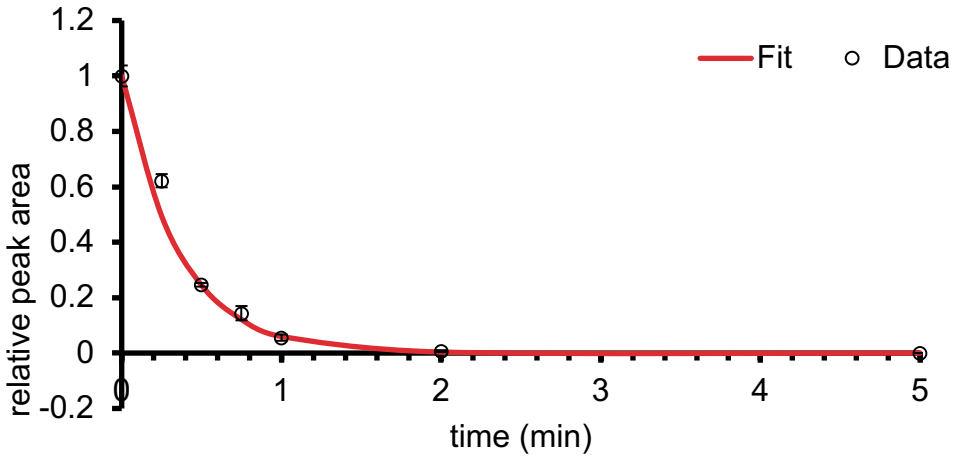


Figure S123. **($t_{1/2}$ = 0.25 ± 0.03 min)** Proteolysis of **23Q** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

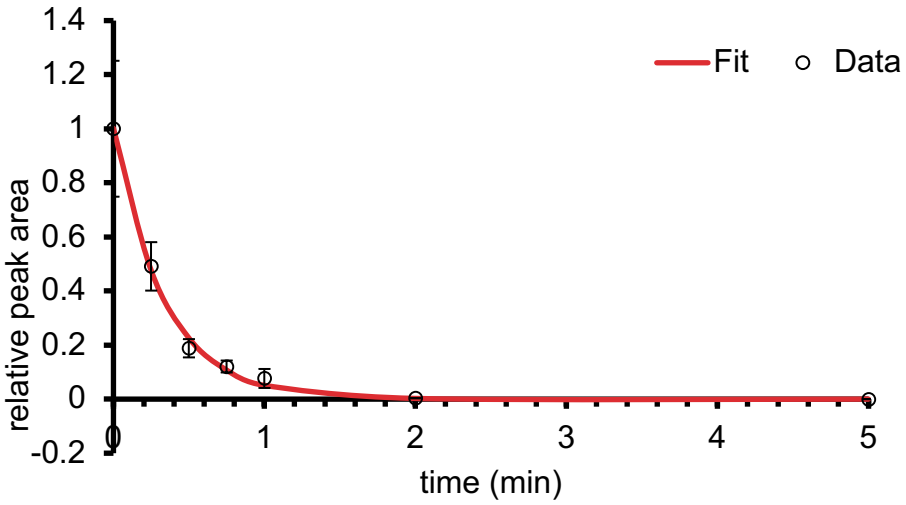


Figure S124. **($t_{1/2}$ = 0.23 ± 0.01 min)** Proteolysis of **23Qp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

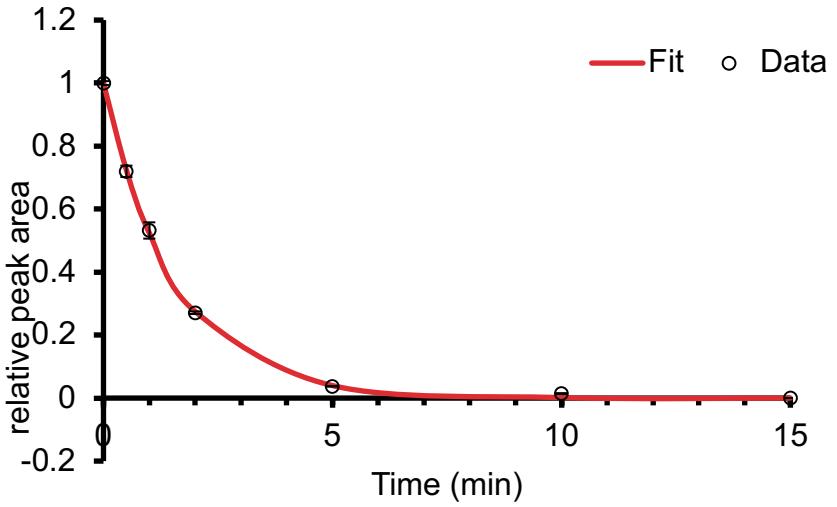


Figure S125. **($t_{1/2}$ = 1.07 ± 0.02 min)** Proteolysis of **27Q** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

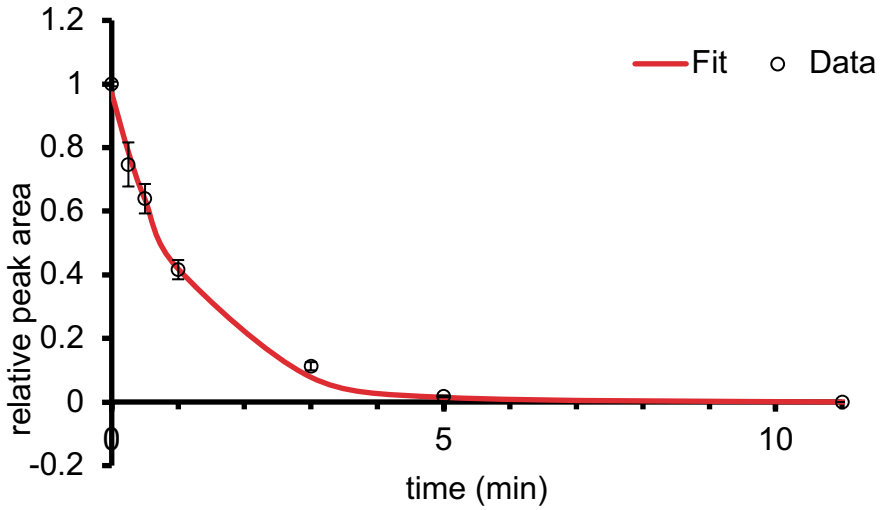


Figure S126. **($t_{1/2}$ = 0.82 ± 0.05 min)** Proteolysis of **27Qp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

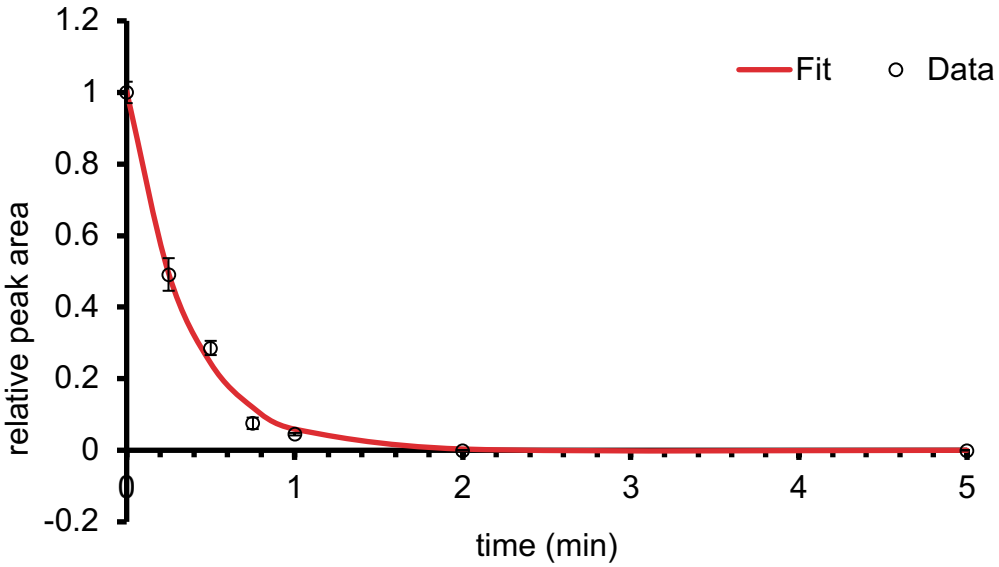


Figure S127. **($t_{1/2}$ = 0.24 ± 0.01 min)** Proteolysis of **29Q** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

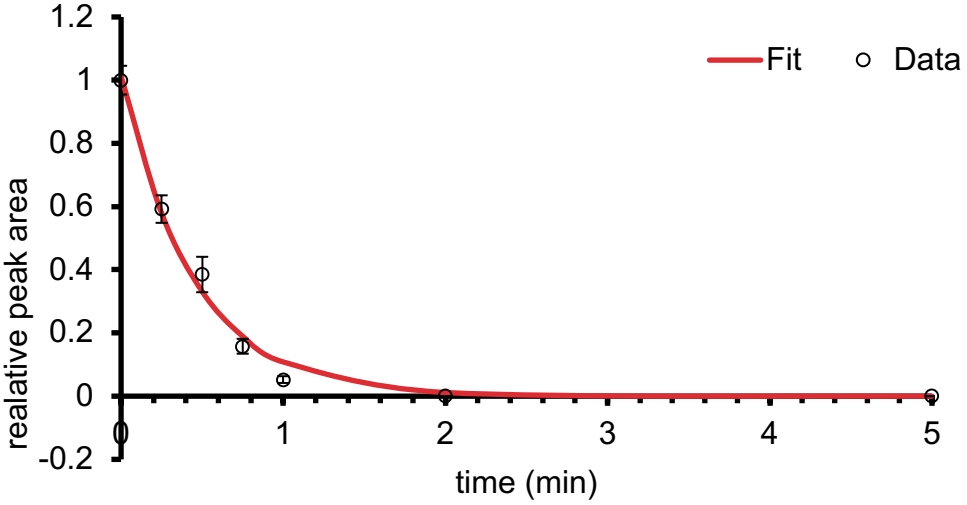


Figure S128. **($t_{1/2}$ = 0.31 ± 0.02 min)** Proteolysis of **29Qp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

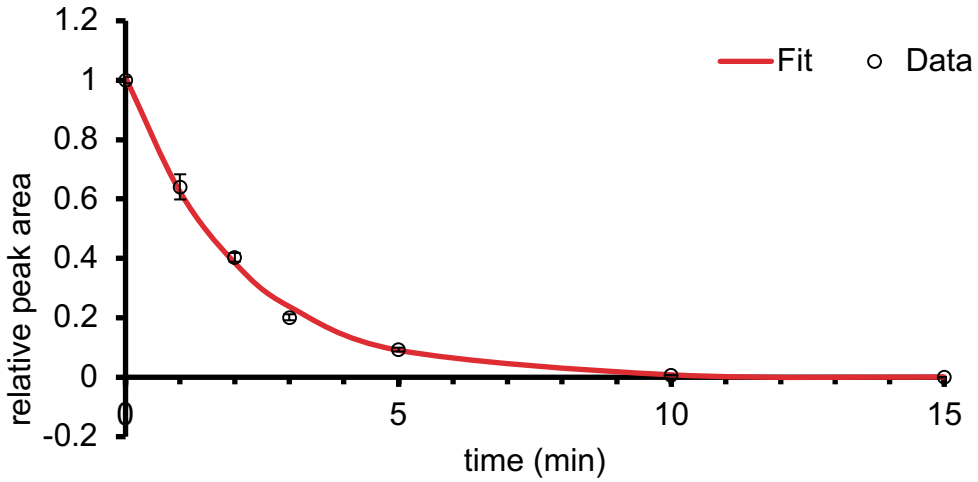


Figure S129. **($t_{1/2}$ = 1.44 ± 0.05 min)** Proteolysis of **32Q** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

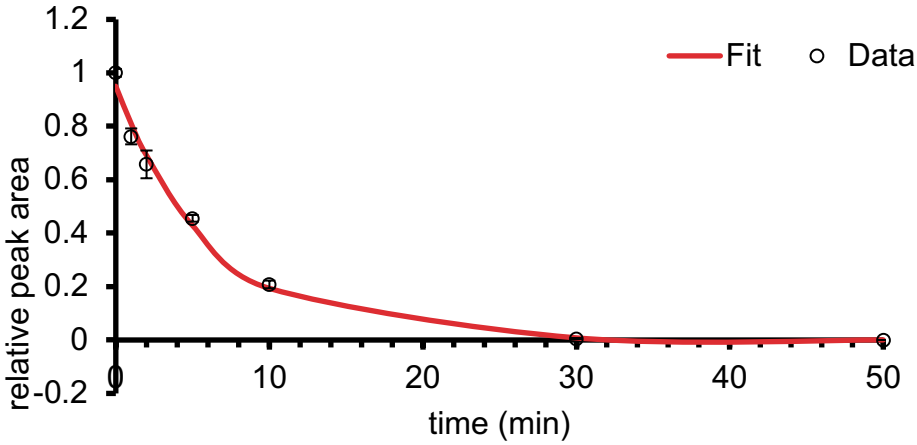


Figure S130. **($t_{1/2}$ = 4.36 ± 0.38 min)** Proteolysis of **32Qp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

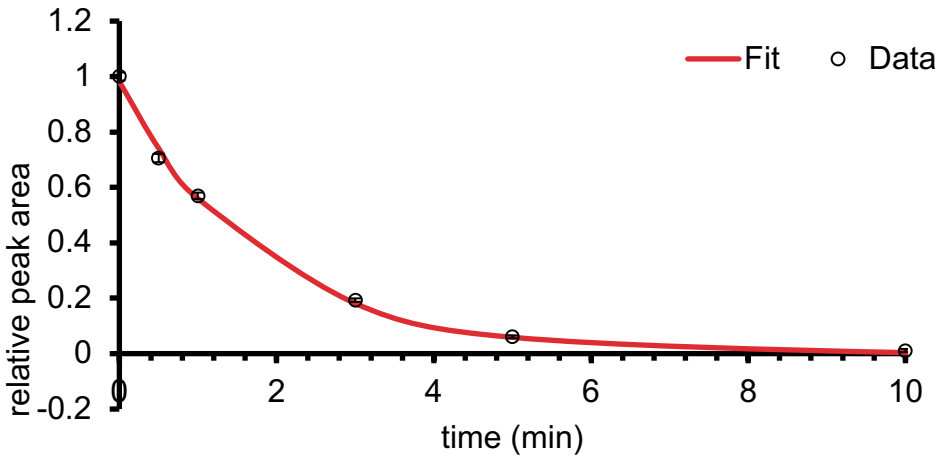


Figure S131. **($t_{1/2}$ = 1.23 ± 0.06 min)** Proteolysis of **16N** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

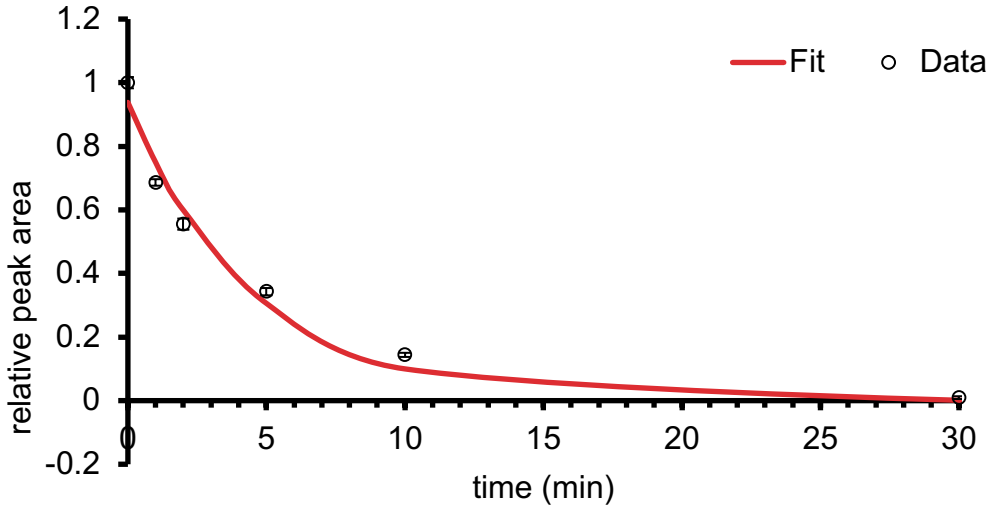


Figure S132. **($t_{1/2}$ = 3.09 ± 0.38 min)** Proteolysis of **16Np** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

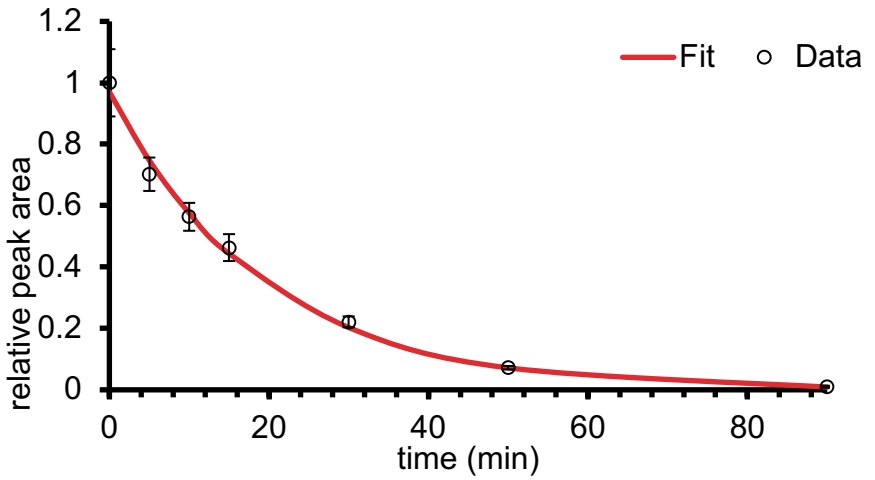


Figure S133. **($t_{1/2}$ = 13.30 ± 0.75 min)** Proteolysis of **16Nbp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

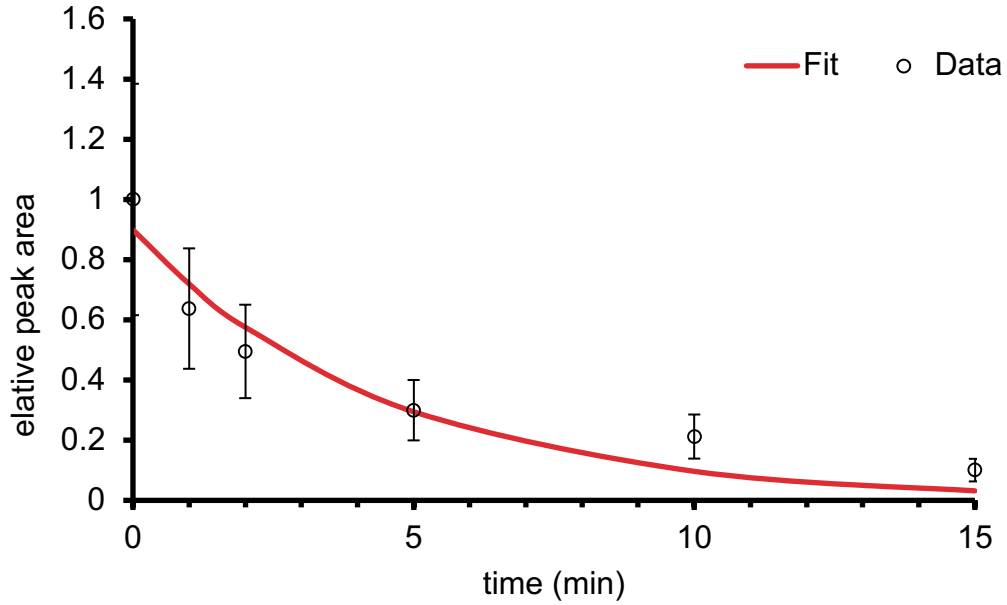


Figure S134. **($t_{1/2}$ = 3.11 ± 0.88 min)** Proteolysis of **18N** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

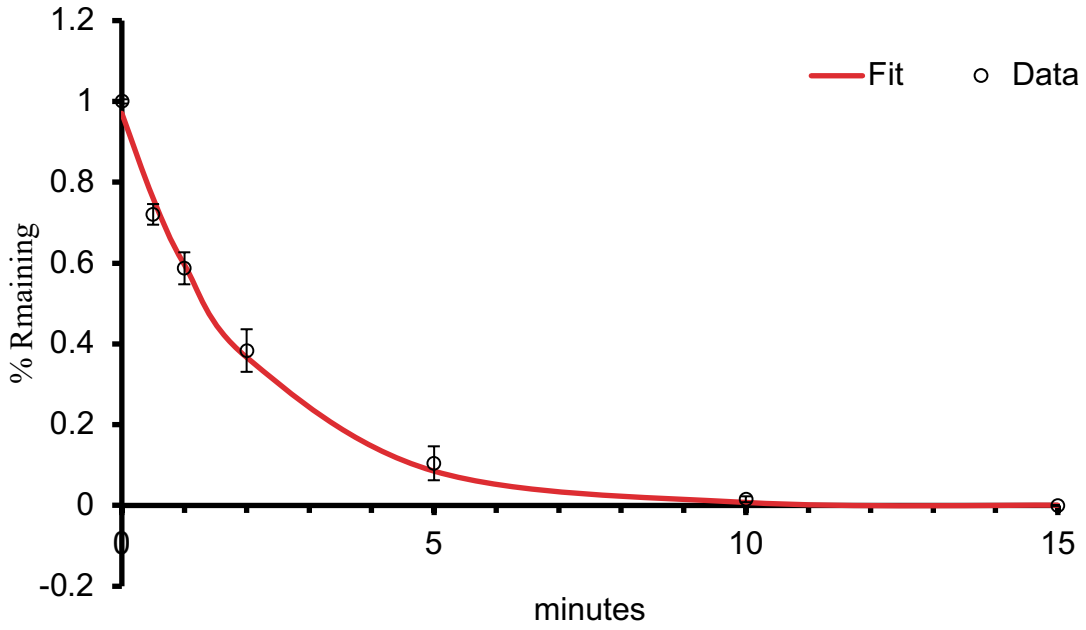


Figure S135. **($t_{1/2}$ = ± min)** Proteolysis of **18Np** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

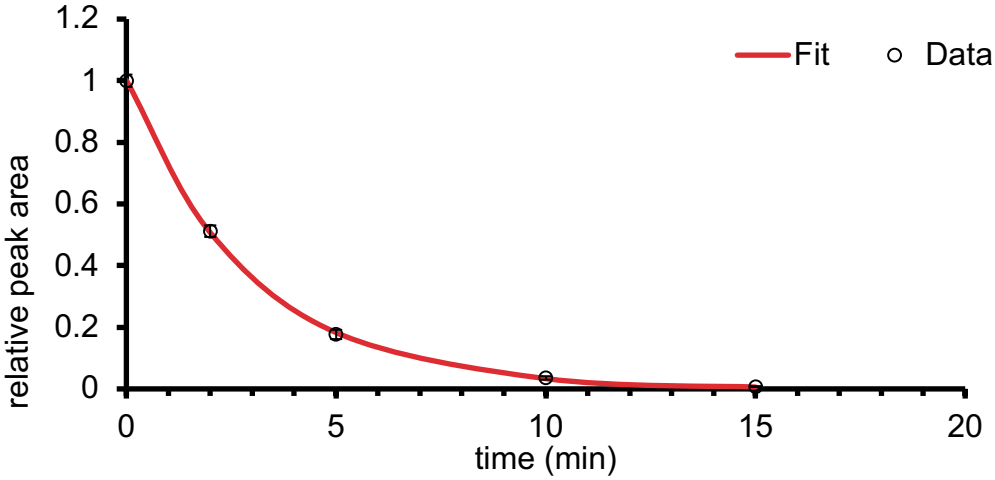


Figure S136. **($t_{1/2}$ = ± min)** Proteolysis of **18Nbp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

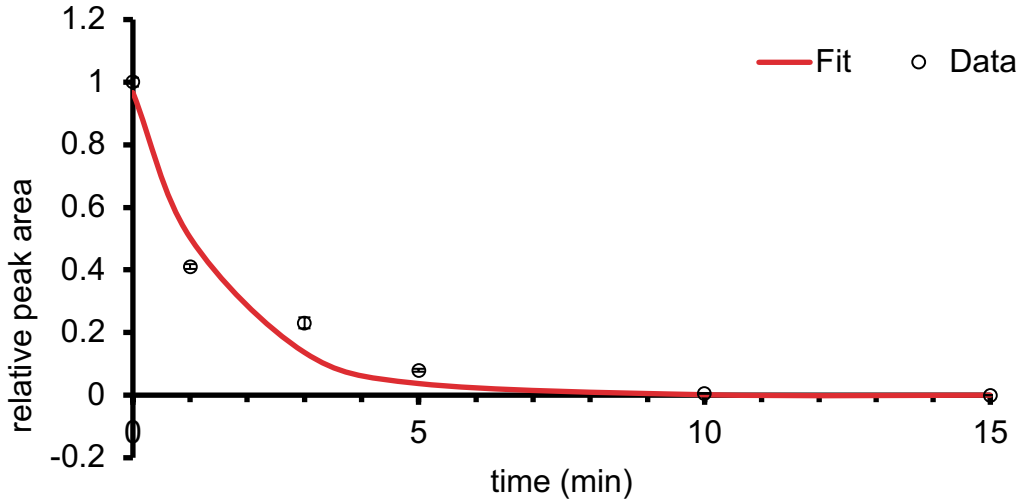


Figure S137. **($t_{1/2}$ = 1.06 ± 0.19 min)** Proteolysis of **19N** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

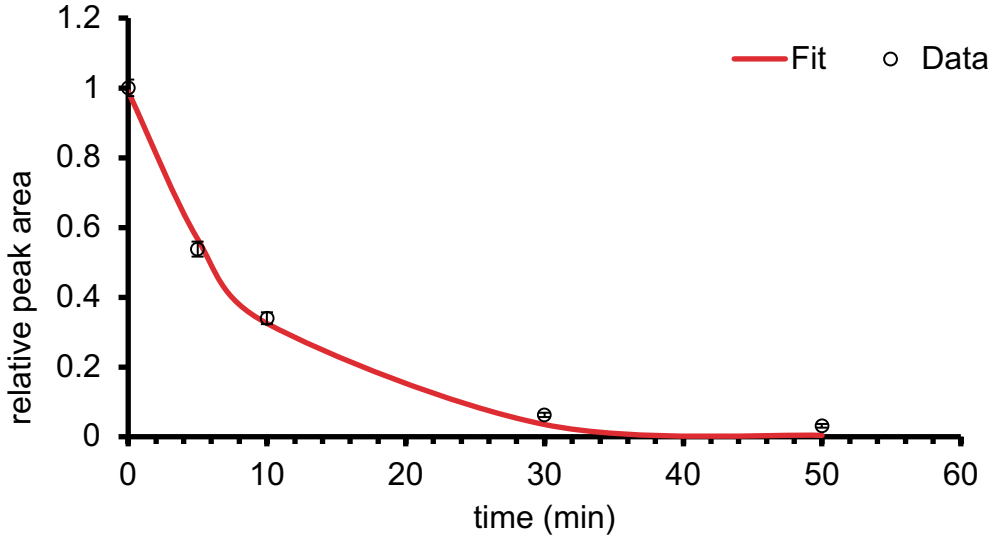


Figure S138. **($t_{1/2}$ = 6.22 ± 0.44 min)** Proteolysis of **19Np** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

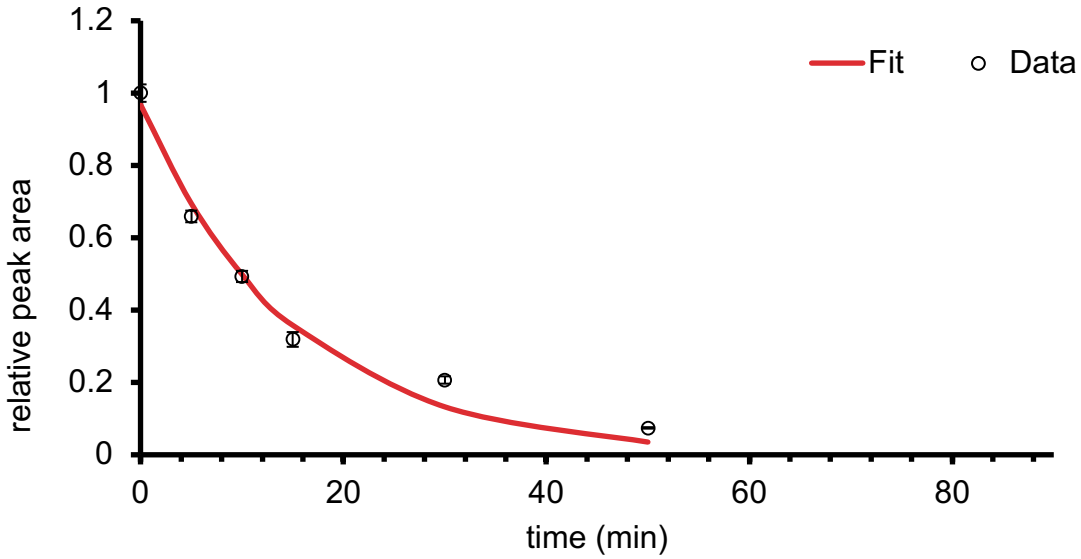


Figure S139. **($t_{1/2}$ = 10.45 ± 1.12 min)** Proteolysis of **19Nbp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

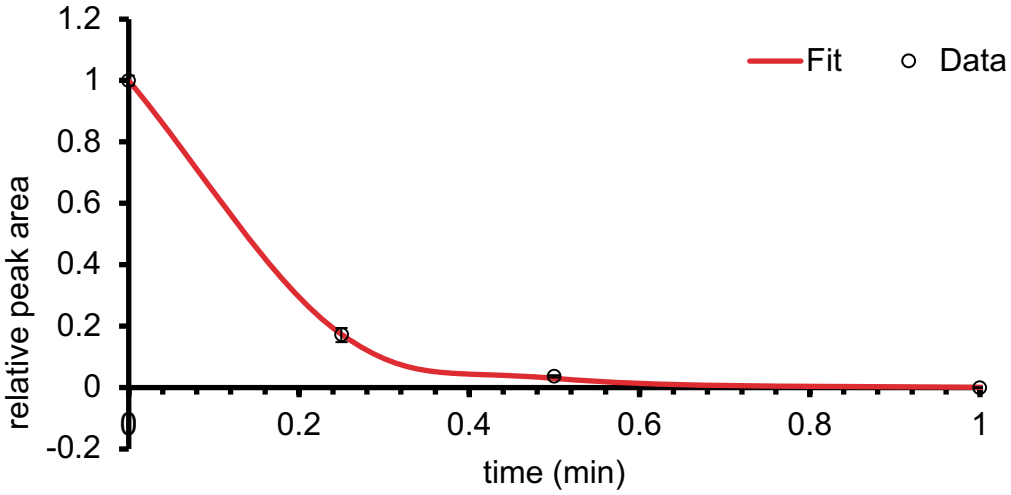


Figure S140. **($t_{1/2}$ = 0.010 ± 0.001 min)** Proteolysis of **23N** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

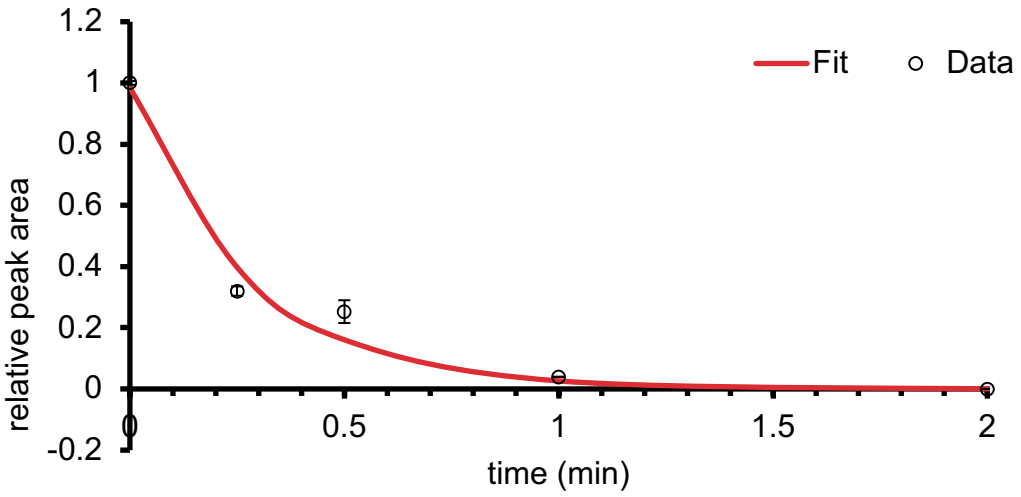


Figure S141. **($t_{1/2}$ = 0.19 ± 0.03 min)** Proteolysis of **23Np** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

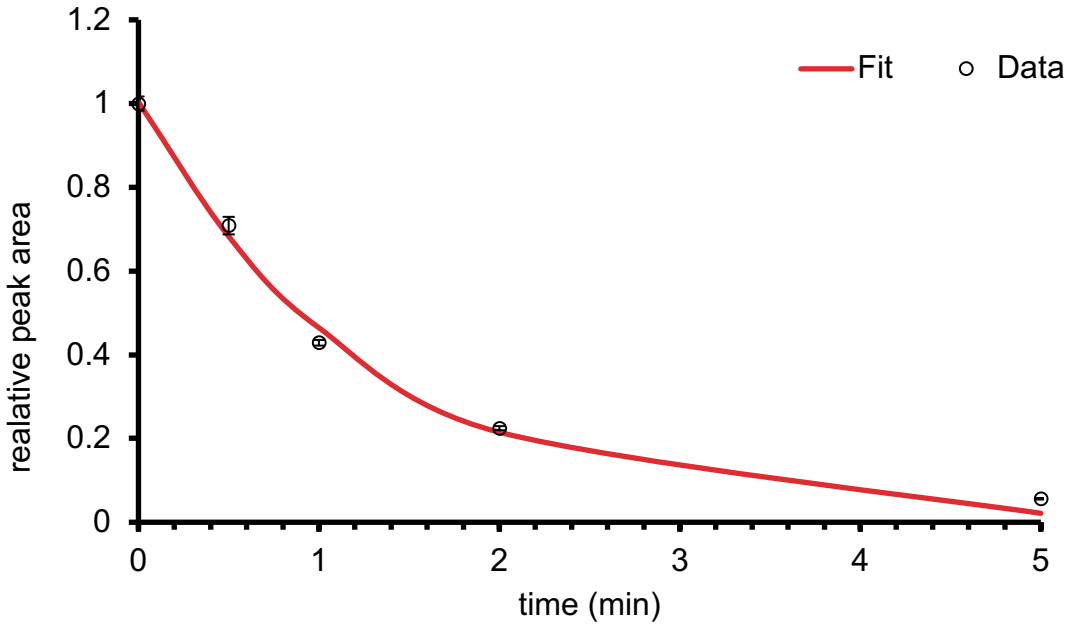


Figure S142. **($t_{1/2}$ = 0.90 ± 0.06 min)** Proteolysis of **23Nbp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

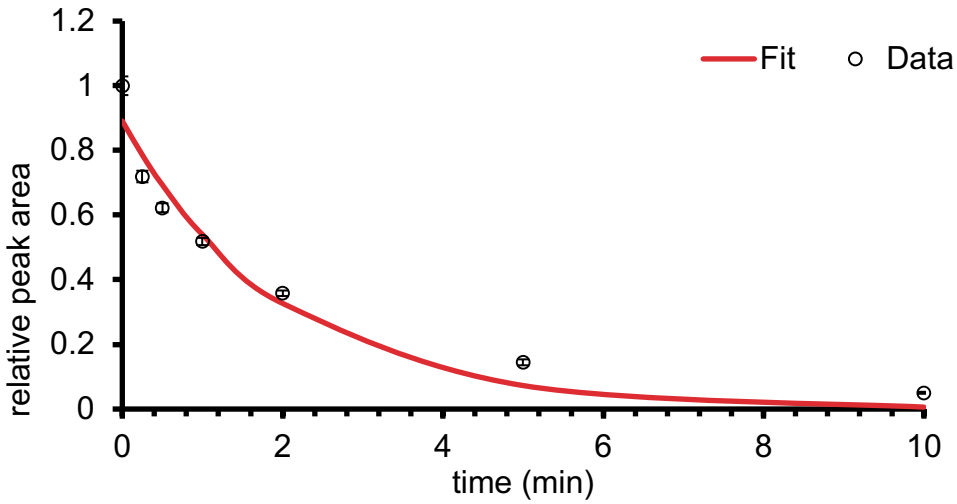


Figure S143. **($t_{1/2}$ = 1.38 ± 0.30 min)** Proteolysis of **27N** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

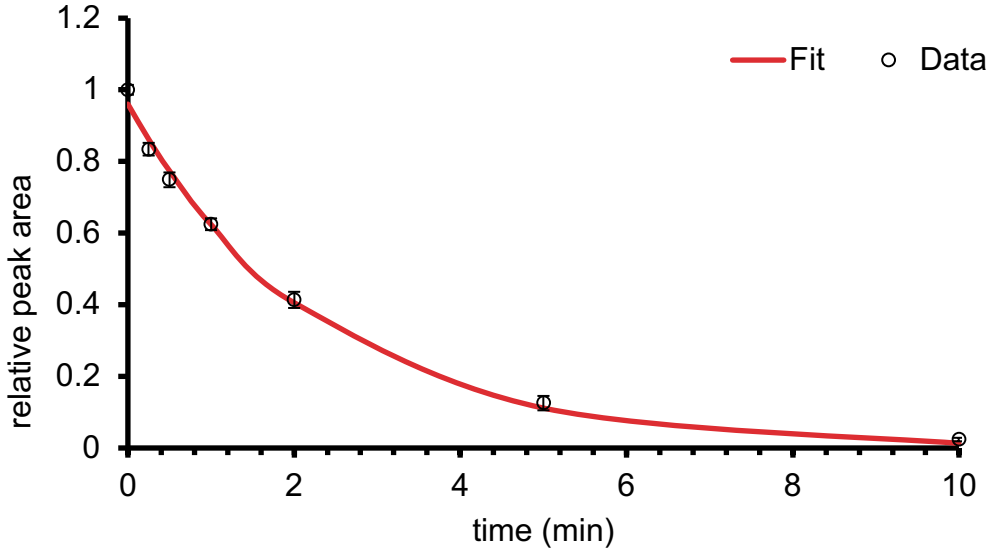


Figure S144. **($t_{1/2}$ = 1.61 ± 0.11 min)** Proteolysis of **27Np** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

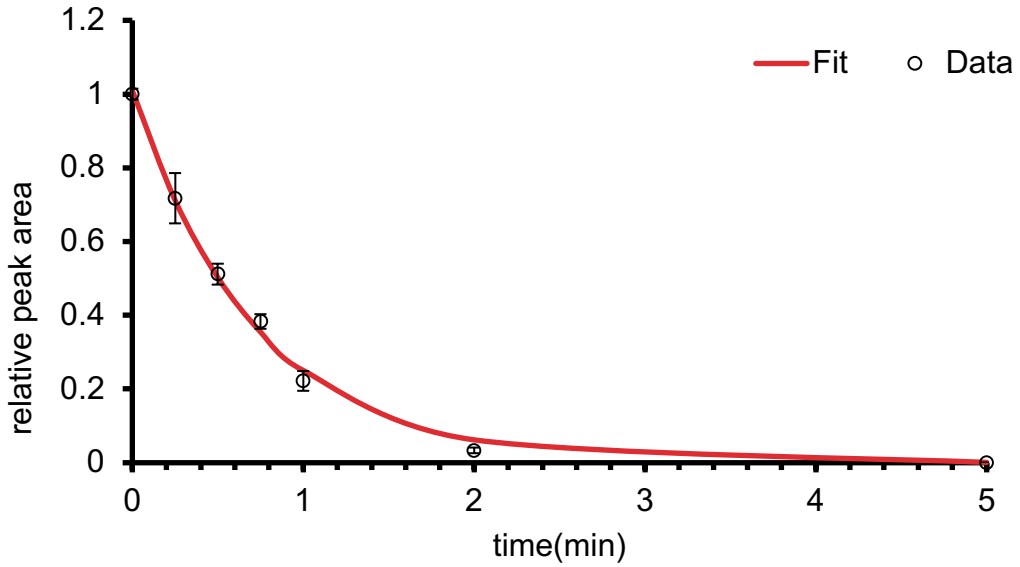


Figure S145. **($t_{1/2}$ = 0.50 ± 0.02 min)** Proteolysis of **27Nbp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

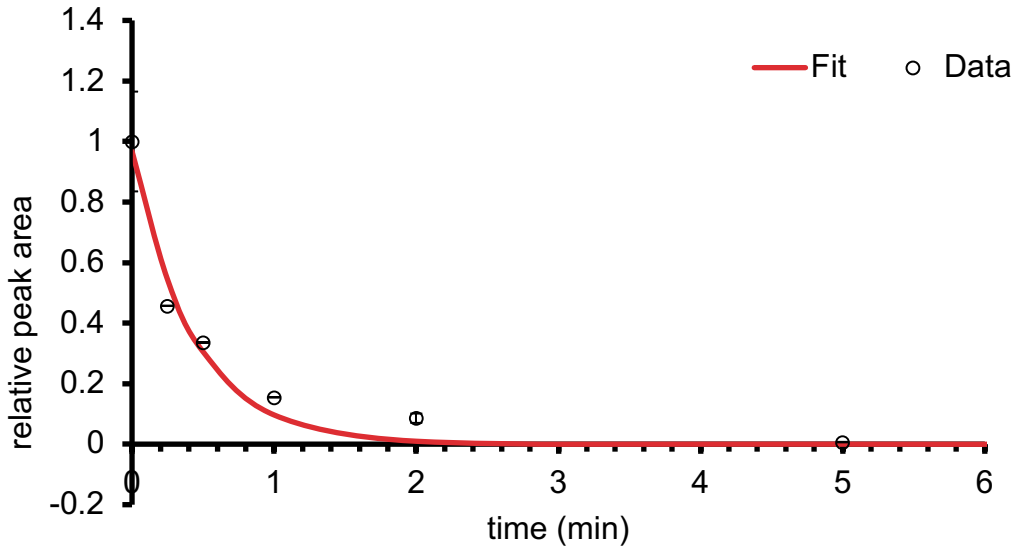


Figure S146. **($t_{1/2}$ = 0.30 ± 0.04 min)** Proteolysis of **29N** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

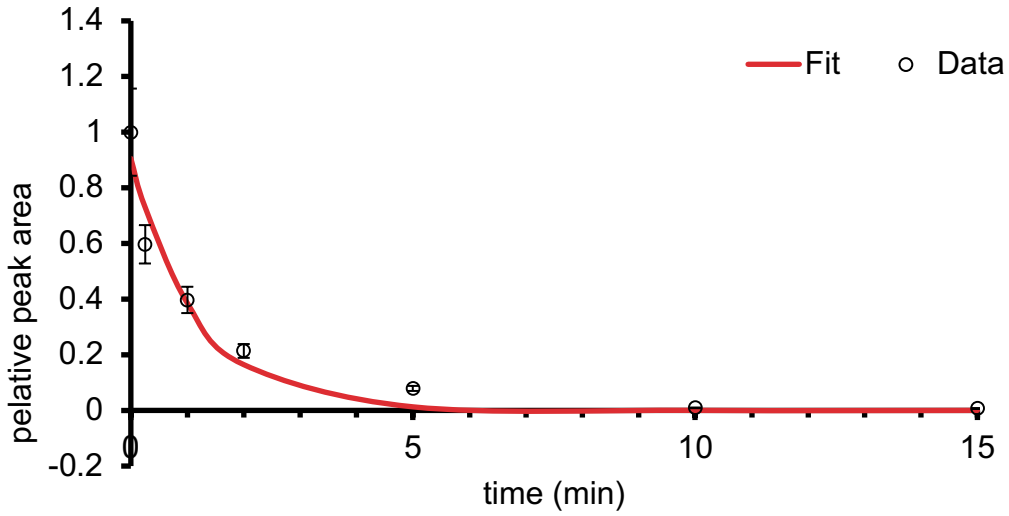


Figure S147. **($t_{1/2}$ = 0.81 ± 0.17 min)** Proteolysis of **29Np** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

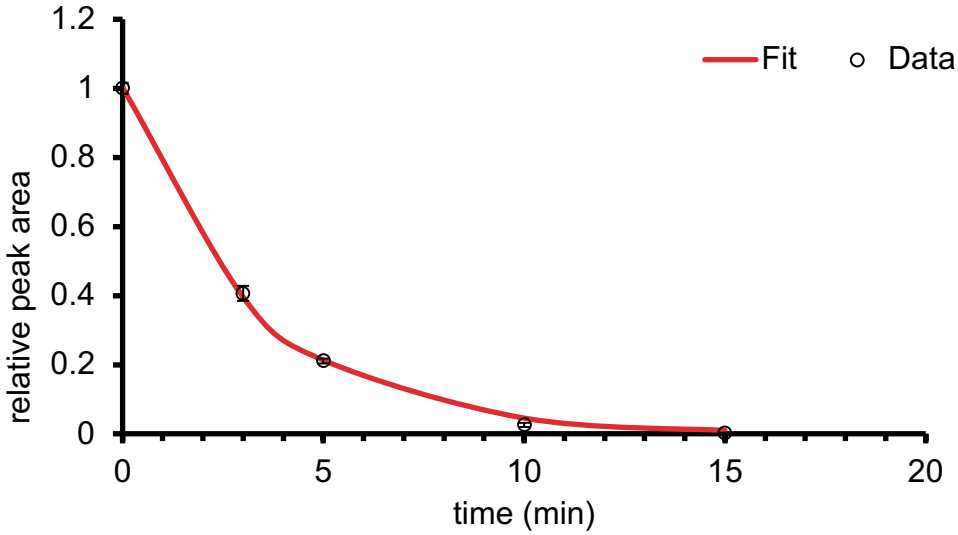


Figure S148. **($t_{1/2}$ = 2.24± 0.06 min)** Proteolysis of **29Nbp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

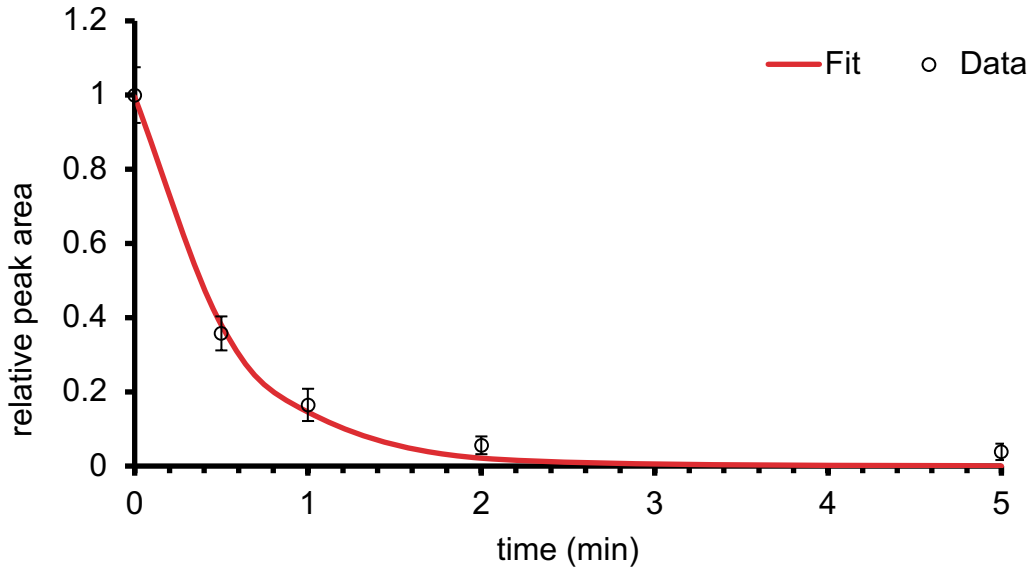


Figure S149. **($t_{1/2}$ = 0.36± 0.03 min)** Proteolysis of **32N** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

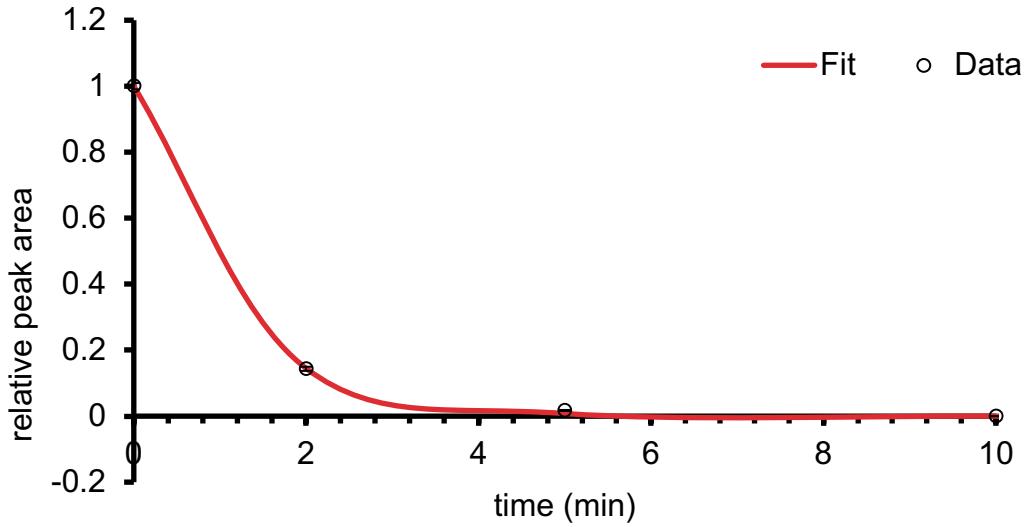


Figure S150. **($t_{1/2}$ = 0.72± 0.01 min)** Proteolysis of **32Np** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

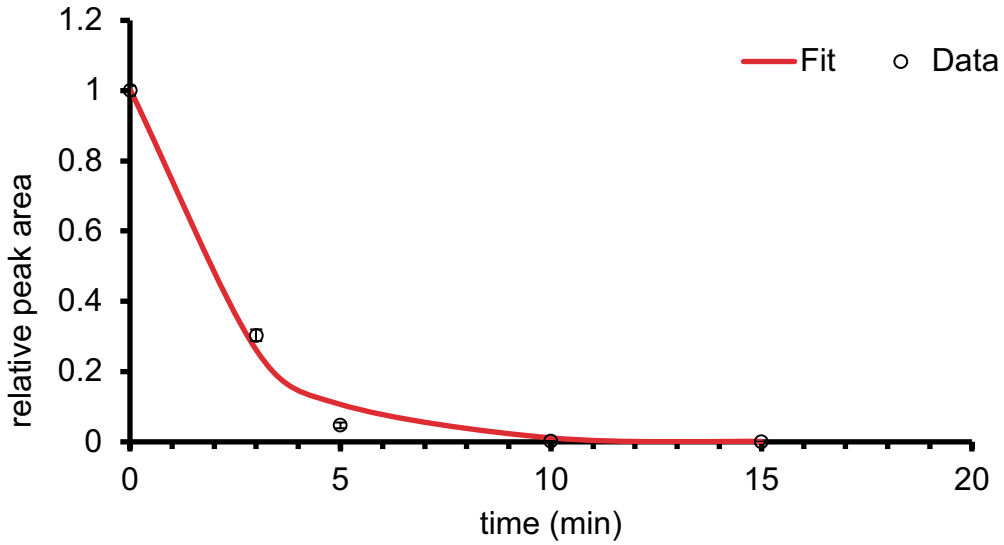


Figure S151. **($t_{1/2}$ = 1.54 ± 0.16 min)** Proteolysis of **32Nbp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

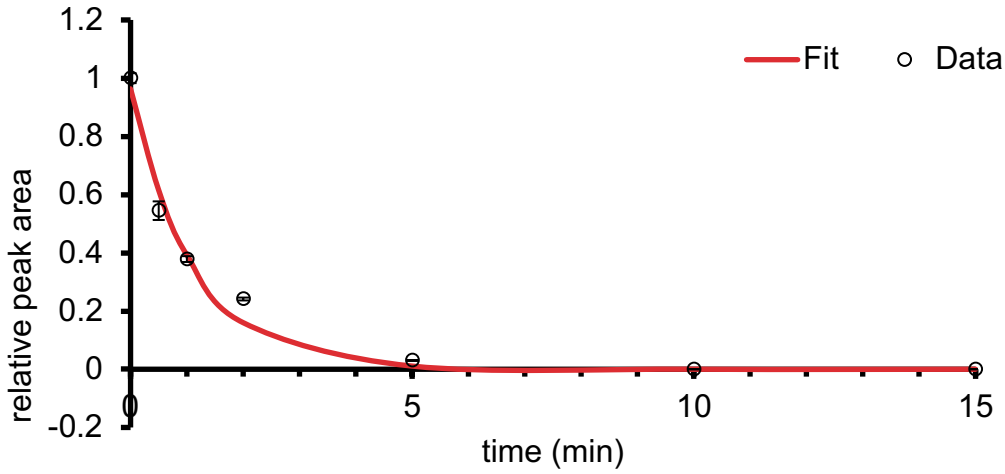


Figure S152. **($t_{1/2}$ = 0.77 ± 0.09 min)** Proteolysis of **16X** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

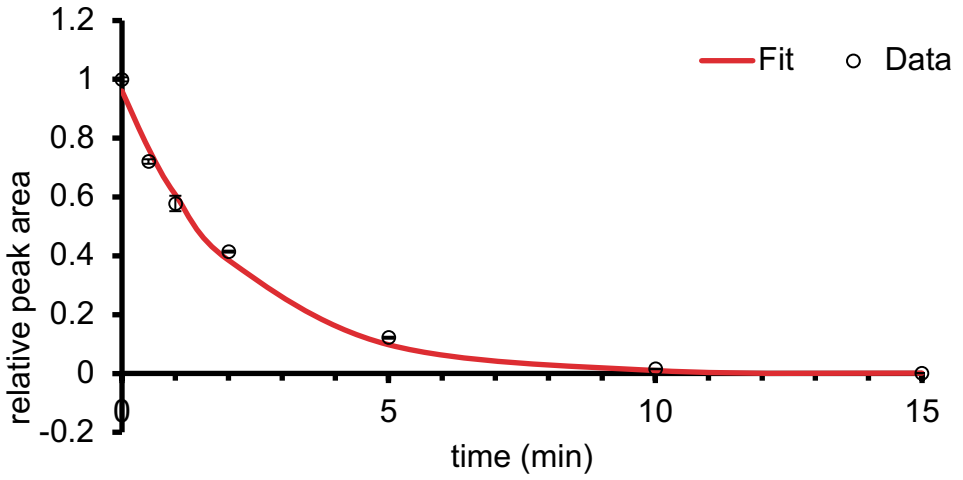


Figure S153. **($t_{1/2}$ = 1.52 ± 0.12 min)** Proteolysis of **16Xp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

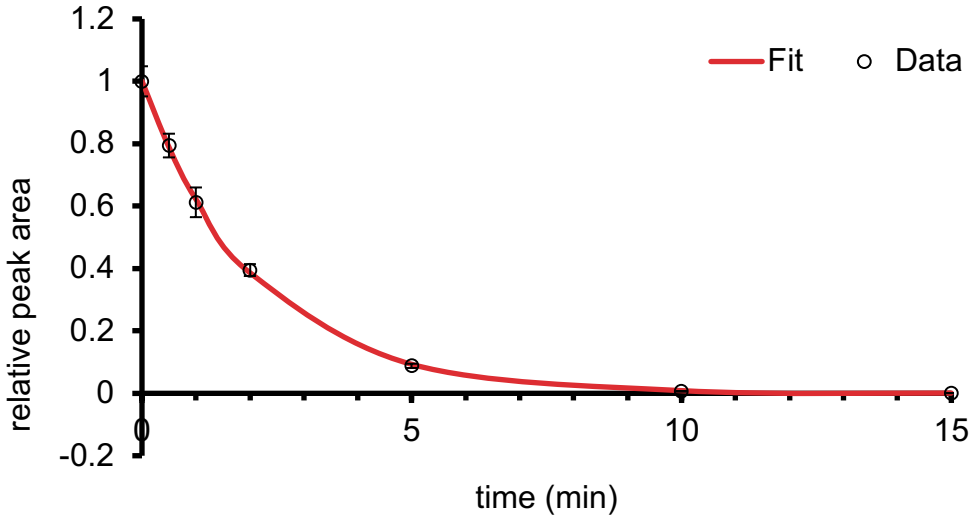


Figure S154. **($t_{1/2}$ = 1.46 ± 0.02 min)** Proteolysis of **18X** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

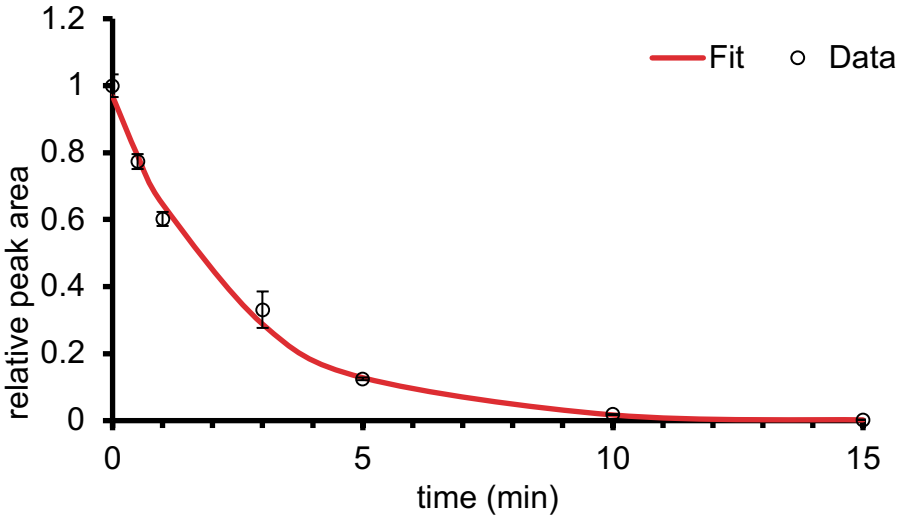


Figure S155. **($t_{1/2}$ = 1.71 ± 0.12 min)** Proteolysis of **18Xp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

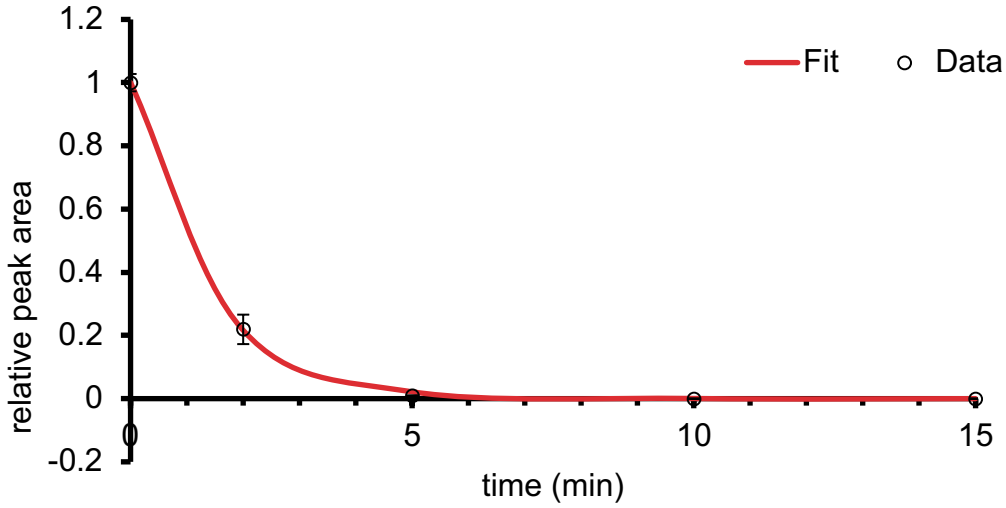


Figure S156. **($t_{1/2}$ = 0.90 ± 0.02 min)** Proteolysis of **19X** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

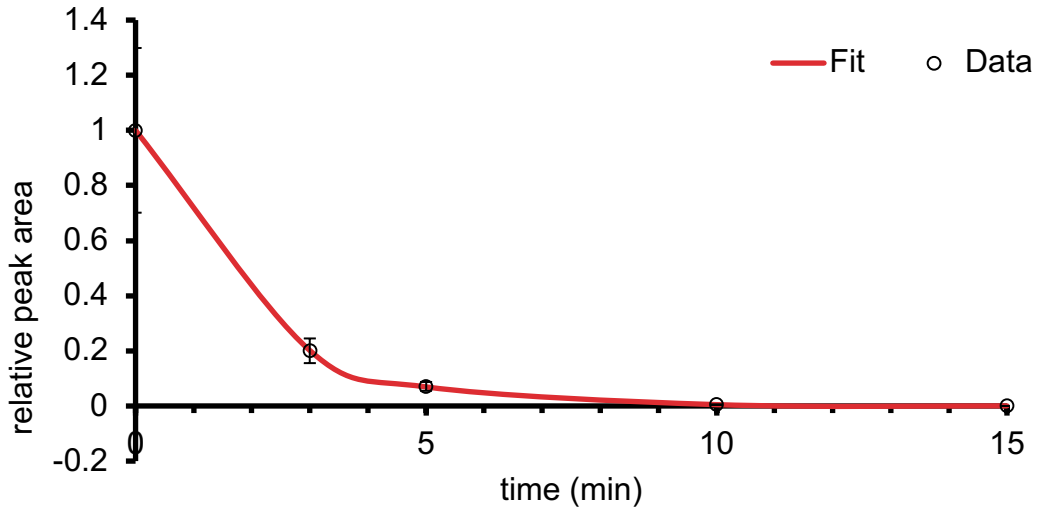


Figure S157. **($t_{1/2}$ = 1.30 ± 0.01 min)** Proteolysis of **19Xp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

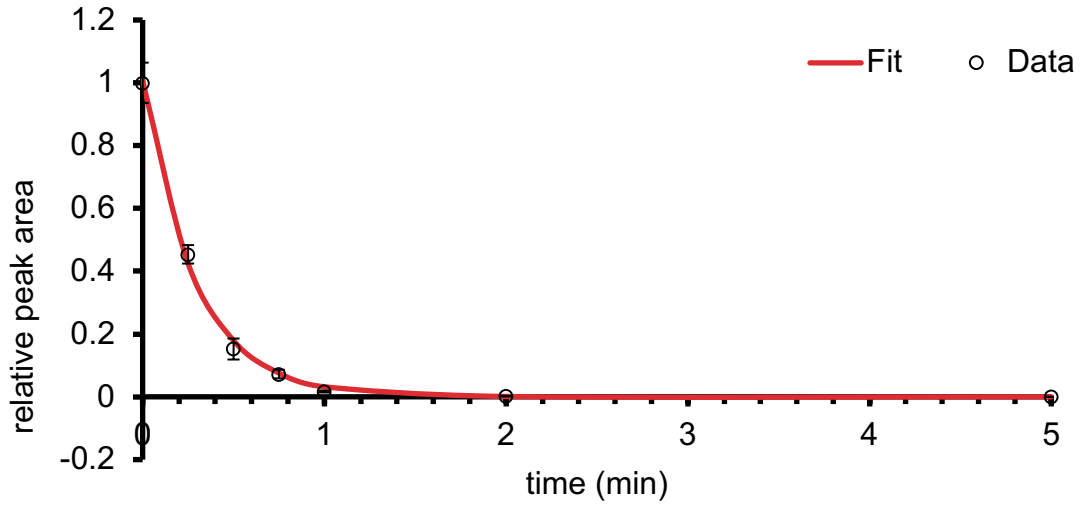


Figure S158. **($t_{1/2}$ = 0.20 ± 0.01 min)** Proteolysis of **23X** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

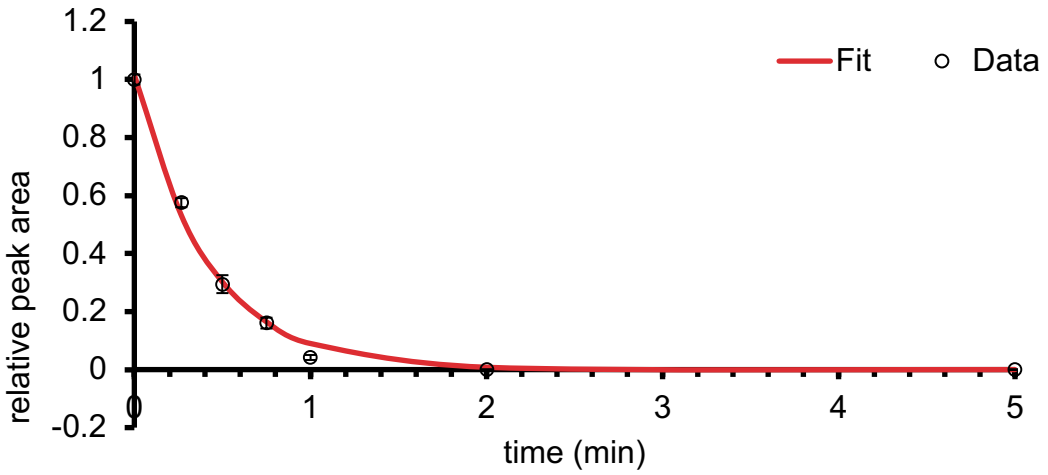


Figure S159. **($t_{1/2}$ = 0.28 ± 0.02 min)** Proteolysis of **23Xp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

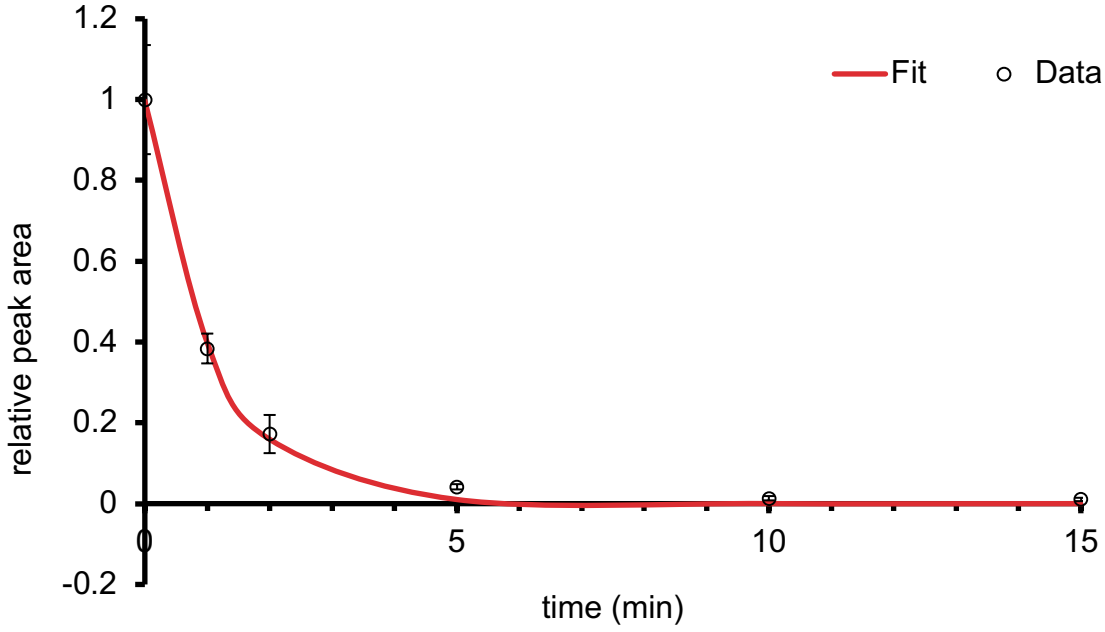


Figure S160. **($t_{1/2}$ = 0.75 ± 0.04 min)** Proteolysis of **27X** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

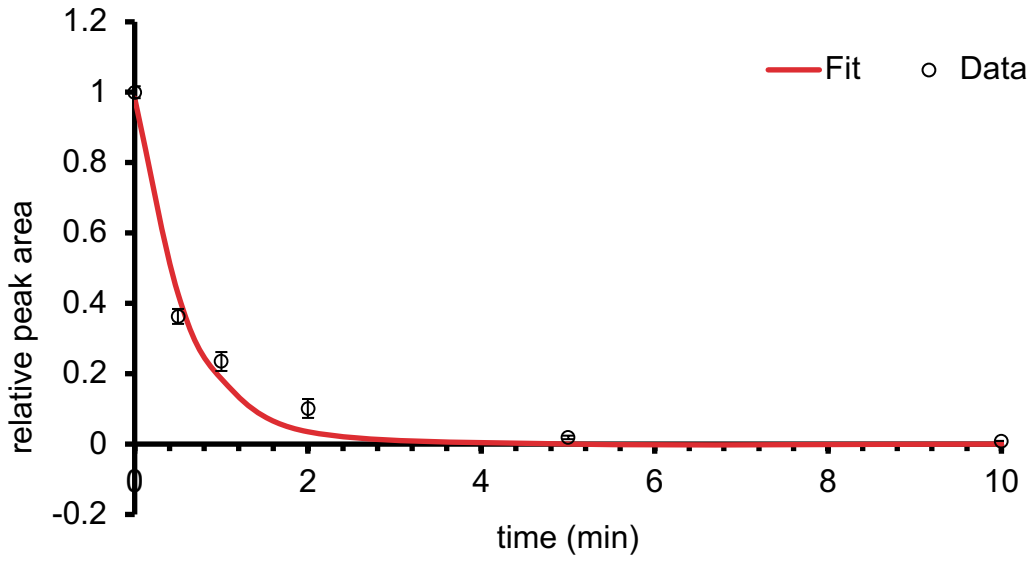


Figure S161. **($t_{1/2}$ = 0.42 ± 0.05 min)** Proteolysis of **27X** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

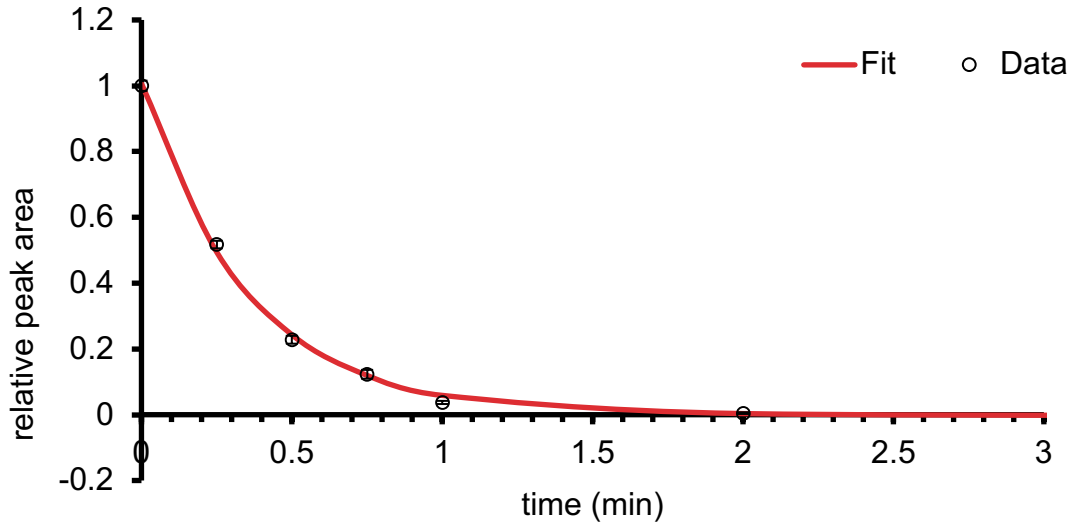


Figure S162. **($t_{1/2}$ = 0.24 ± 0.01 min)** Proteolysis of **29X** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

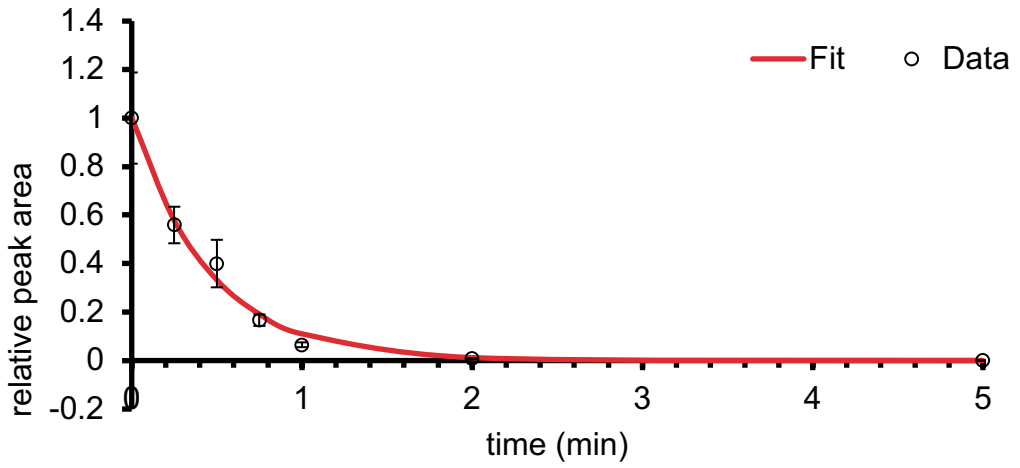


Figure S163. **($t_{1/2}$ = 0.31 ± 0.02 min)** Proteolysis of **29Xp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

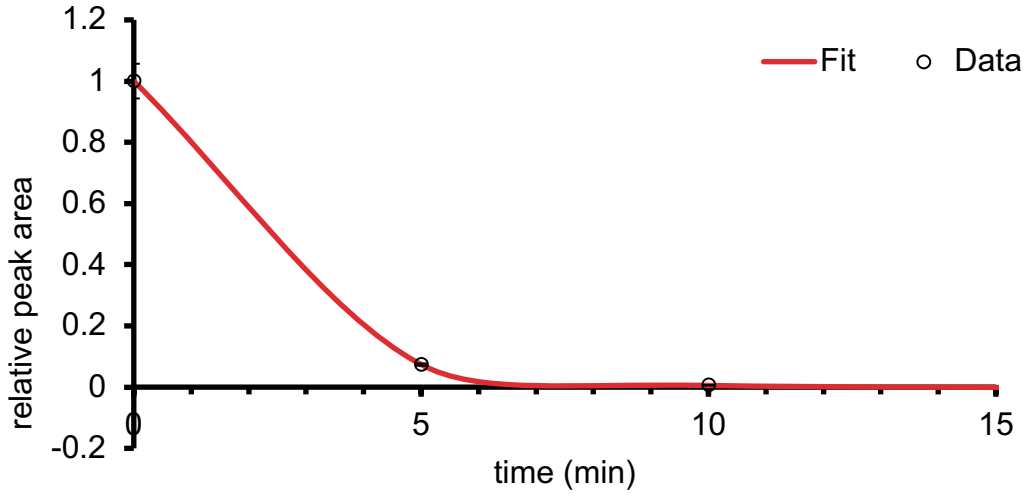


Figure S164. **($t_{1/2}$ = 1.33 ± 0.02 min)** Proteolysis of **32X** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

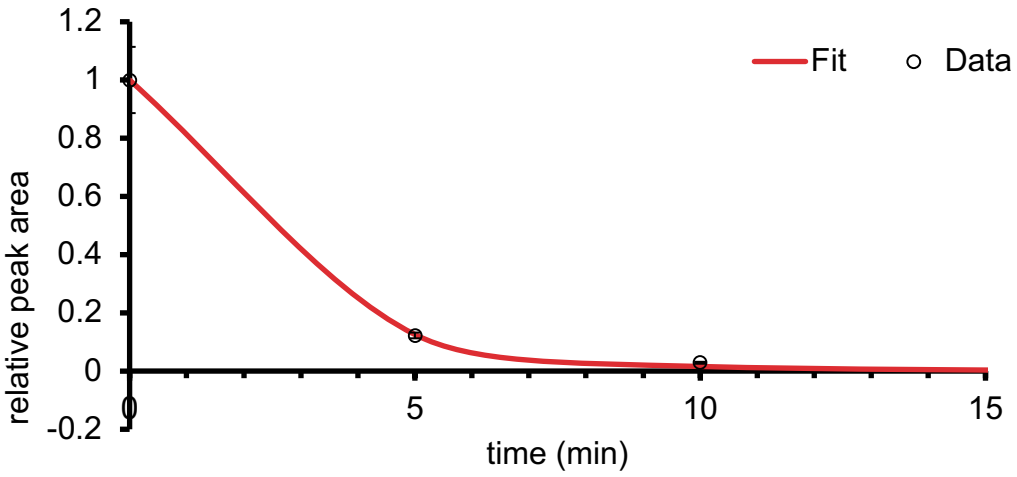


Figure S165. **($t_{1/2}$ = 1.67 ± 0.04 min)** Proteolysis of **32Xp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

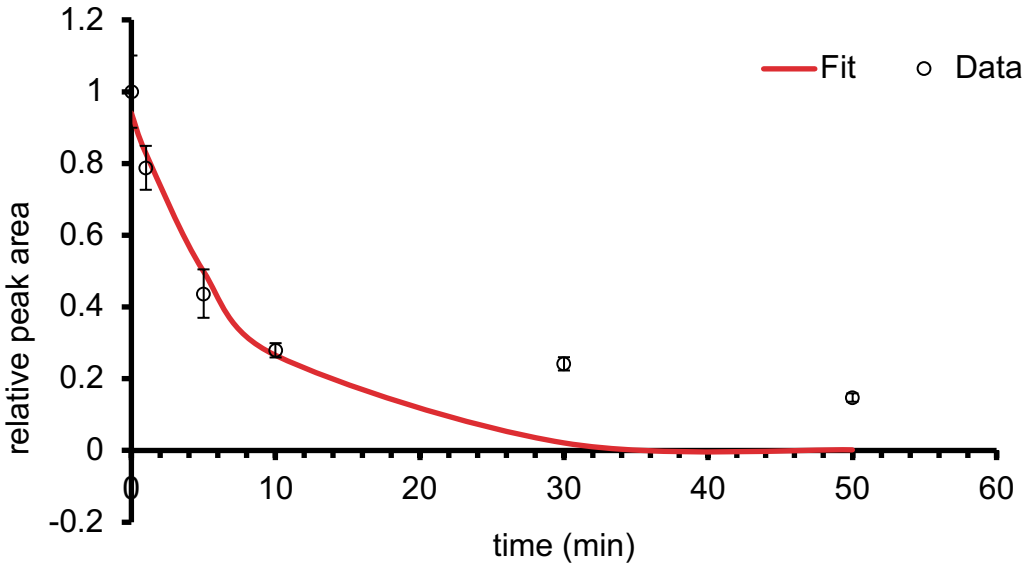


Figure S166. **($t_{1/2}$ = 5.48 ± 1.86 min)** Proteolysis of **16Z** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

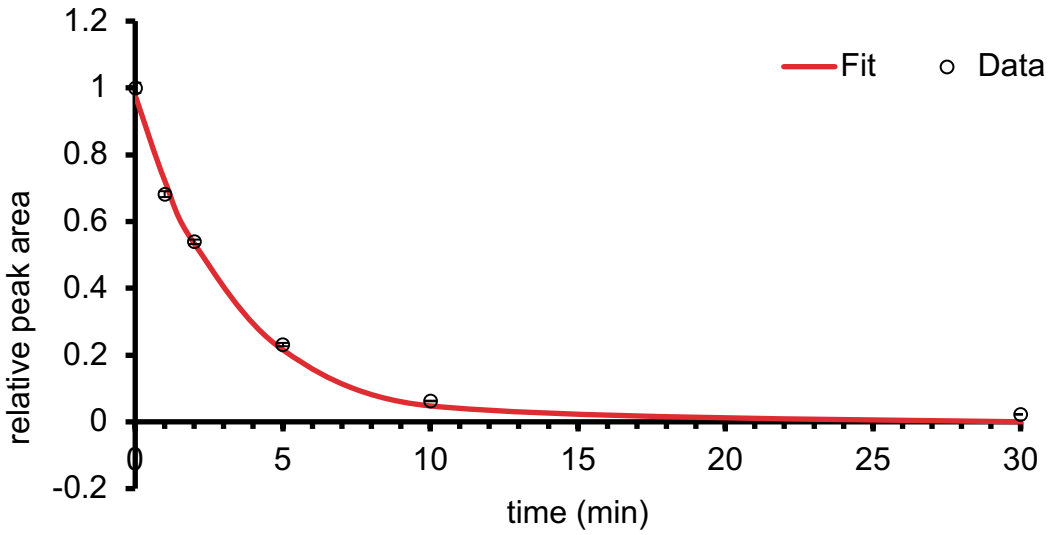


Figure S167. **($t_{1/2}$ = 1.67 ± 0.04 min)** Proteolysis of **16Zp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

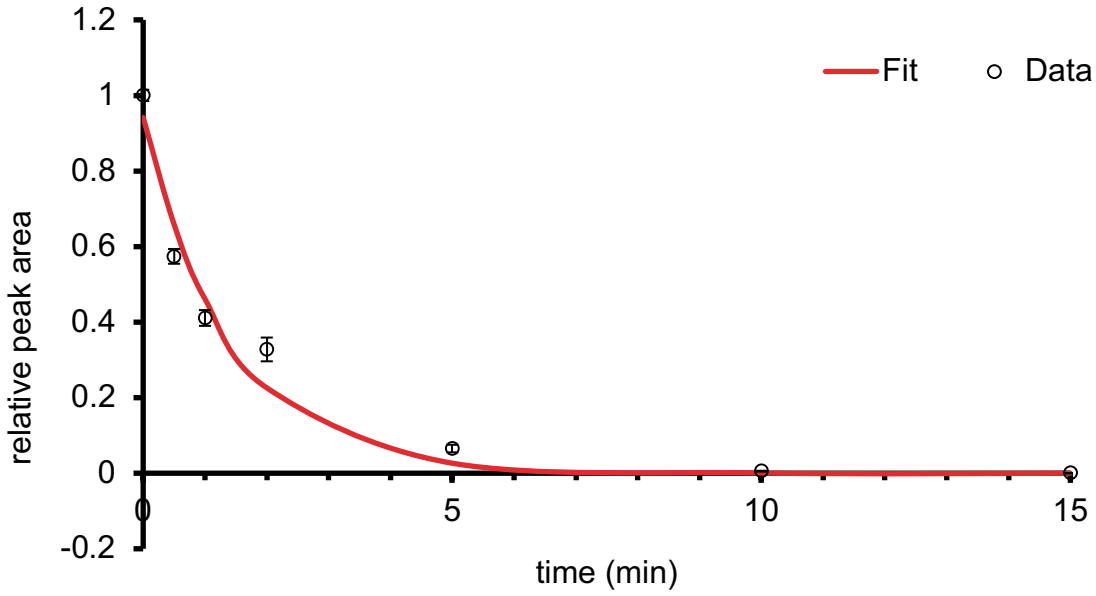


Figure S167. **($t_{1/2}$ = 0.97 ± 0.16 min)** Proteolysis of **18Z** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

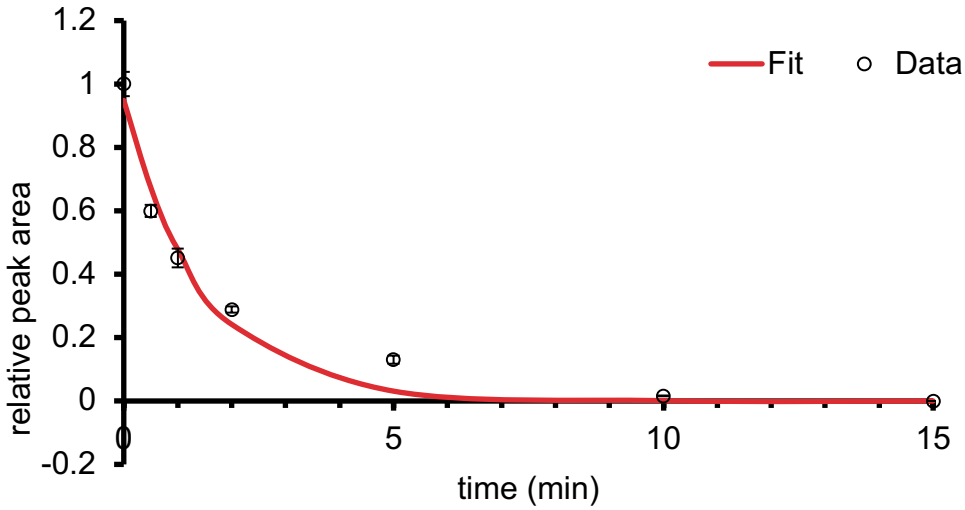


Figure S168. **($t_{1/2}$ = 1.01 ± 0.15 min)** Proteolysis of **18Zp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

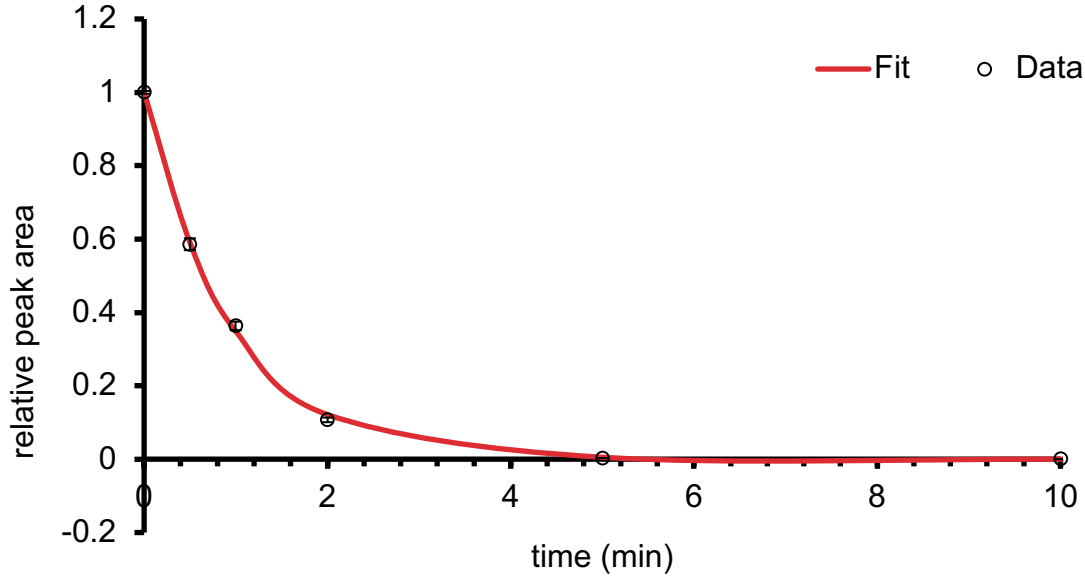


Figure S169. **($t_{1/2}$ = 0.66 ± 0.01 min)** Proteolysis of **19Z** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

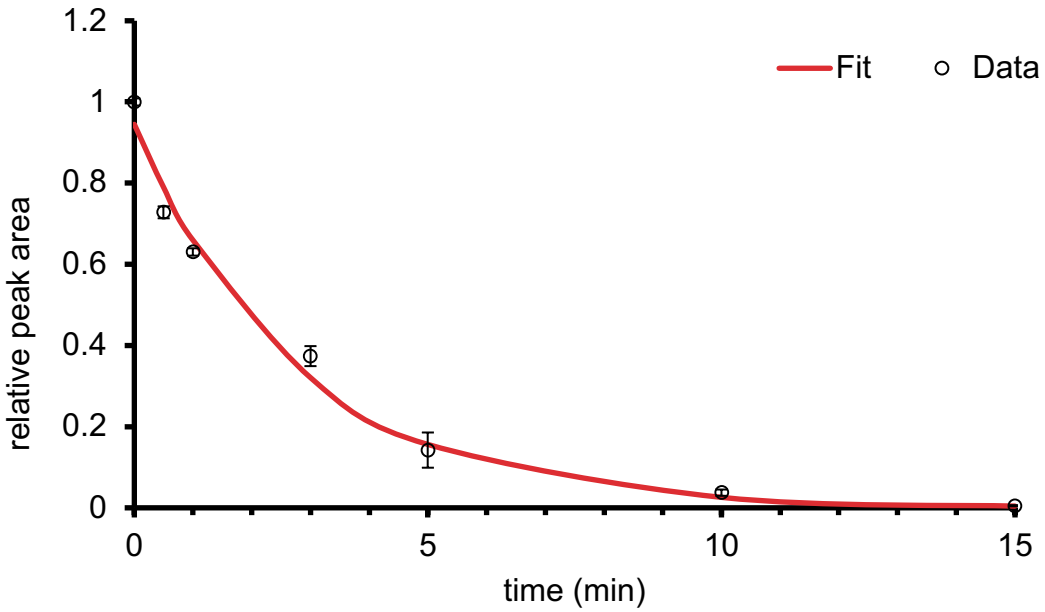


Figure S170. **($t_{1/2}$ = 1.93 ± 0.2 min)** Proteolysis of **19Zp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

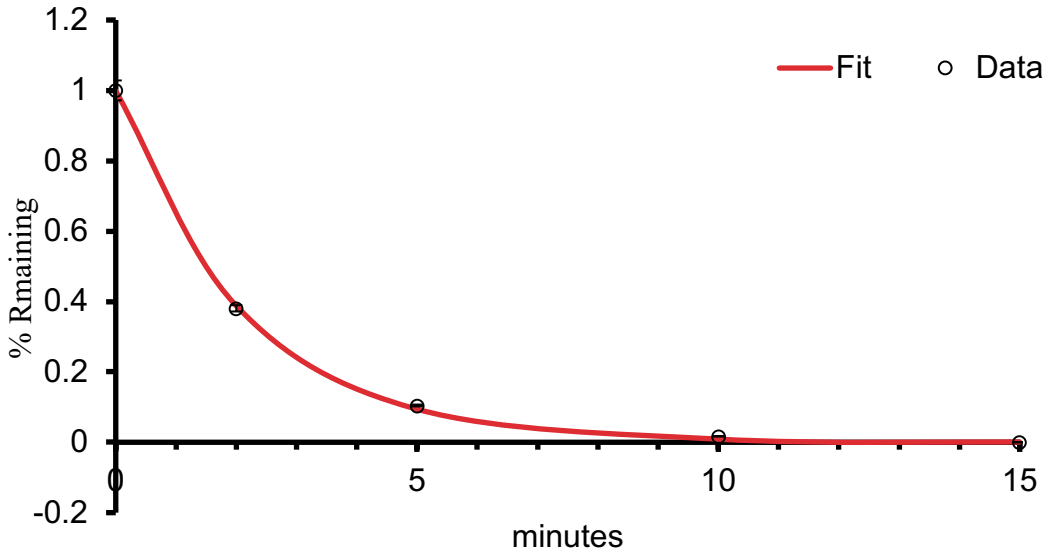


Figure S171. **($t_{1/2}$ = 1.47 ± 0.03 min)** Proteolysis of **23Z** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

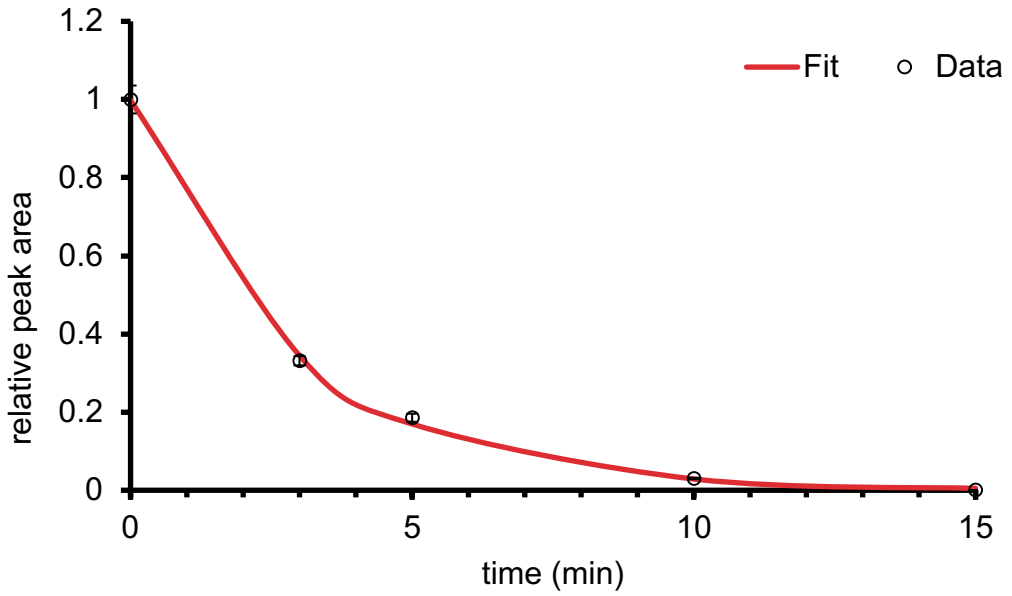


Figure S172. **($t_{1/2}$ = 1.95 ± 0.06 min)** Proteolysis of **23Zp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

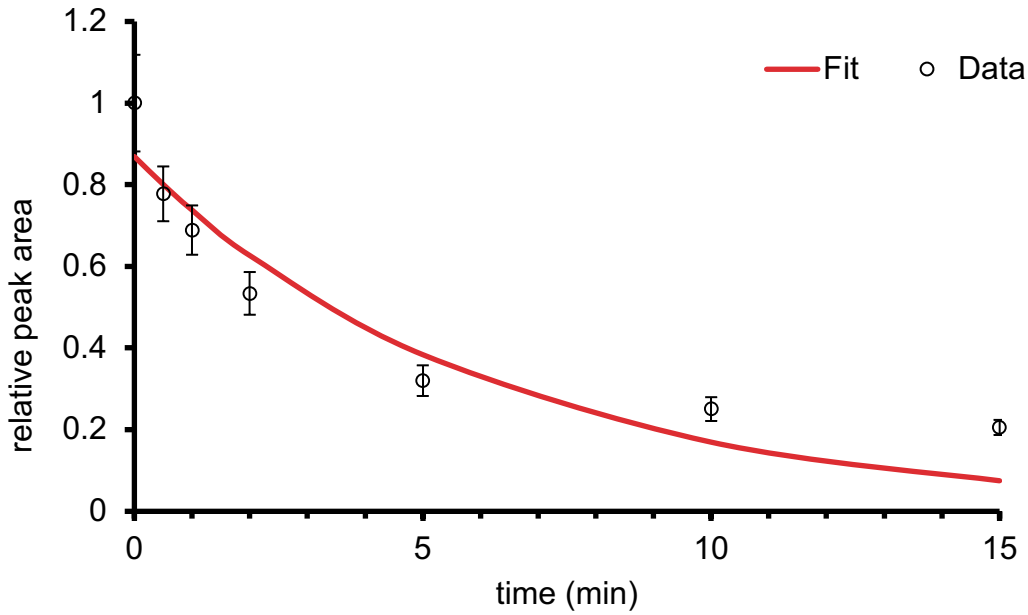


Figure S173. **($t_{1/2}$ = 4.24 ± 1.16 min)** Proteolysis of **27Z** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

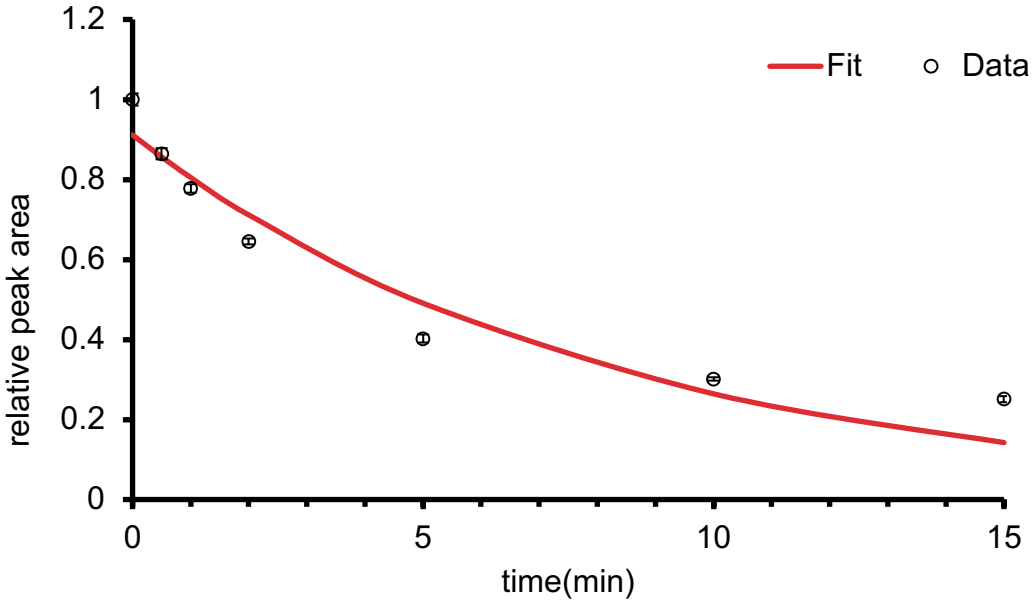


Figure S174. **($t_{1/2}$ = 5.60 ± 1.09 min)** Proteolysis of **27Zp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

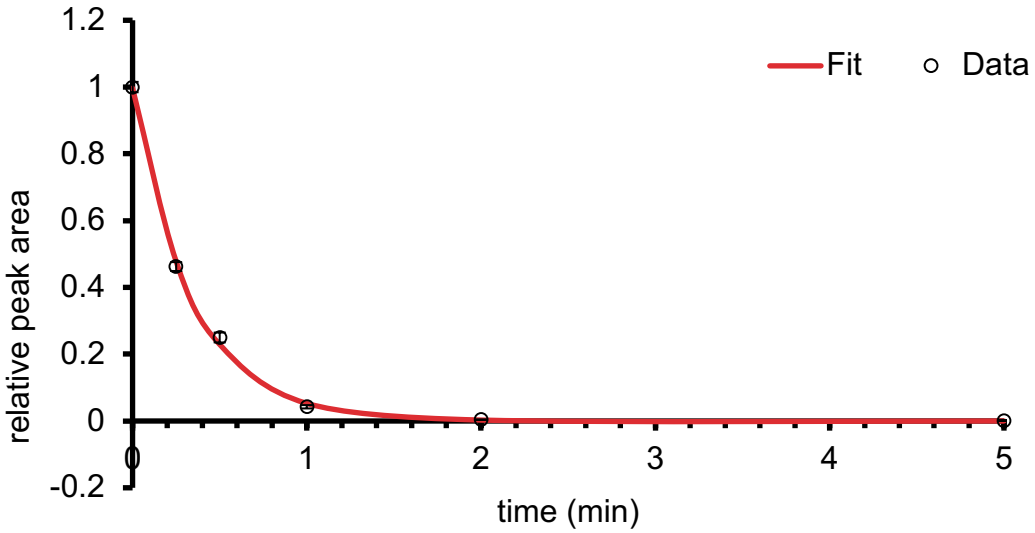


Figure S175. **($t_{1/2}$ = 0.24 ± 0.01 min)** Proteolysis of **29Z** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

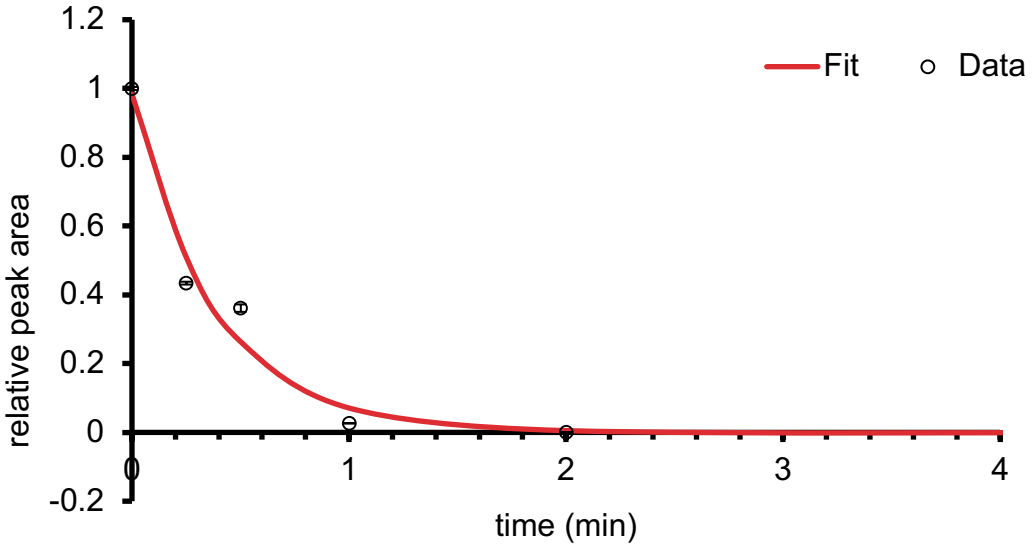


Figure S176. **($t_{1/2}$ = 0.26 ± 0.04 min)** Proteolysis of **29Zp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

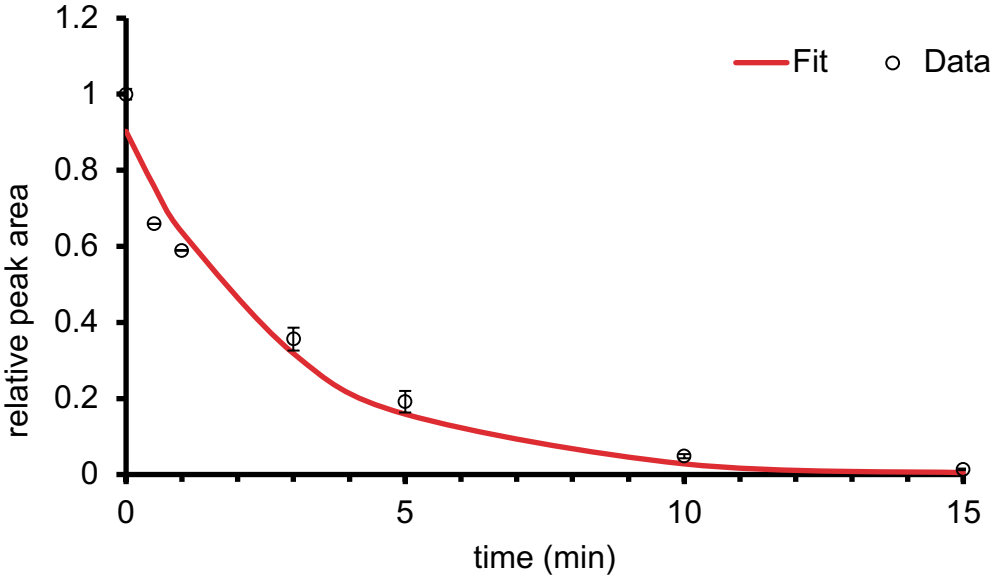


Figure S177. **($t_{1/2}$ = 1.99 ± 0.33 min)** Proteolysis of **32Z** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

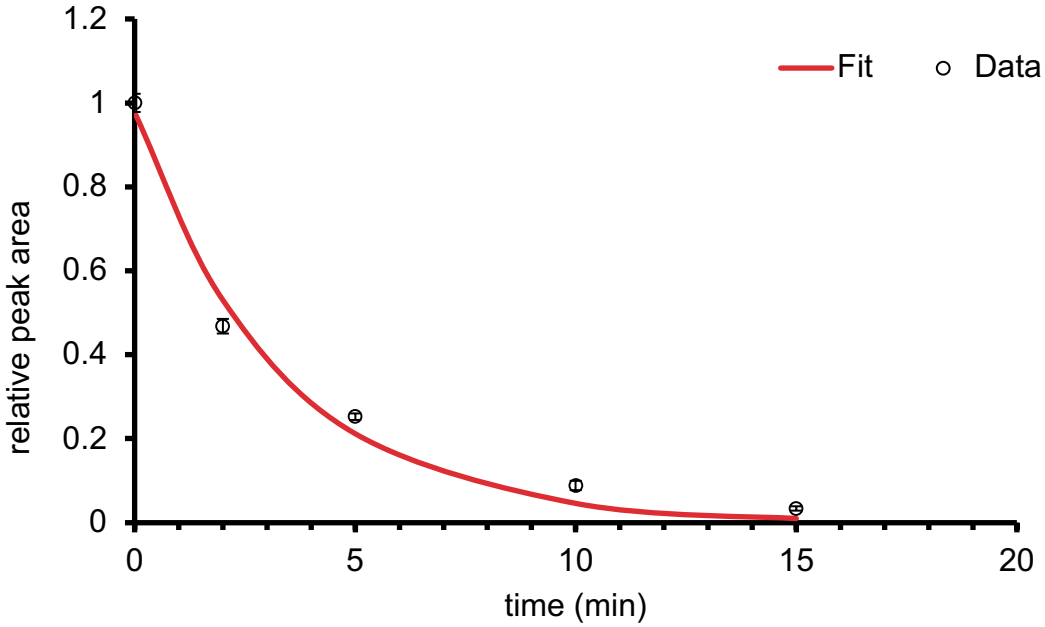


Figure S178. **($t_{1/2}$ = 2.27 ± 0.28 min)** Proteolysis of **32Zp** (50 μM protein concentration in 20 mM sodium phosphate buffer, pH 7) by proteinase K (10 mg/mL), as monitored by HPLC. Data points are shown as black circles with error bars, and each represents the average of three replicate experiments. The solid red line represents the fit of the data to a monoexponential decay function, which was used to calculate the indicated half-lives.

References

(1) Lawrence, P. B., Gavrilov, Y., Matthews, S. S., Langlois, M. I., Shental-Bechor, D., Greenblatt, H. M., Pandey, B. K., Smith, M. S., Paxman, R., Torgerson, C. D., et al. (2014) Criteria for Selecting PEGylation Sites on Proteins for Higher Thermodynamic and Proteolytic Stability. *J. Am. Chem. Soc. 136*, 17547-17560.

(2) Lawrence, P. B., Billings, W. M., Miller, M. B., Pandey, B. K., Stephens, A. R., Langlois, M. I., and Price, J. L. (2016) Conjugation Strategy Strongly Impacts the Conformational Stability of a PEG-Protein Conjugate. *ACS Chem. Biol. 11*, 1805–1809.

(3) Garofalo, A., Parat, A., Bordeianu, C., Ghobril, C., Kueny-Stotz, M., Walter, A., Jouhannaud, J., Begin-Colin, S., and Felder-Flesch, D. (2014) Efficient synthesis of small-sized phosphonated dendrons: potential organic coatings of iron oxide nanoparticles. *New J. Chem. 38*, 5226–5239.

(4) Deiters, A., Cropp, T. A., Mukherji, M., Chin, J. W., Anderson, J. C., and Schultz, P. G. (2003) Adding Amino Acids with Novel Reactivity to the Genetic Code of Saccharomyces Cerevisiae. *J. Am. Chem. Soc. 125*, 11782-11783.

(5) Edelhoch, H. (1967) Spectroscopic Determination of Tryptophan and Tyrosine in Proteins. *Biochemistry 6*, 1948–1954.