Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

Controlling Optical and Catalytic Activity of Genetically Engineered Proteins by Ultrasound

*Yu Zhou, Shuaidong Huo, Mark Loznik, Robert Göstl, Arnold J. Boersma, and Andreas Herrmann**

anie_202010324_sm_miscellaneous_information.pdf

Table of Contents

Experimental Procedures

Chemicals

All chemical reagents were of analytical grade and used without further purification. Tryptone and yeast extract used for Terrific Broth (TB) medium were purchased from Duchefa. T4 DNA ligase (5 Weiss U/µL) was purchased from Thermo Fisher Scientific. Roti®GelStain was received from Carl Roth (Germany). The other chemicals were purchased from Sigma Aldrich unless otherwise noted. Ultrapure water with a resistivity > 18.2 MΩ·cm was used for all experiments.

Molecular cloning

Circular permuted GFP (Fig. S1) and BPTI backbone fragments (Fig. S14) with the recognition sites of the restriction enzymes PflMI and BglI were ordered (IDT). The fragments were ligated to pJET 1.2 blunt vector using T4 DNA ligase according to the Blunt-end ligation protocol; the DNA fragment was used in a 3:1 molar ratio with pJET1.2/blunt vector, and the ligation mixture was transformed directly after 5 min at 22 °C incubation. Because BglI is used in further cloning, the present BglI site on the pJET vector was removed using the primers FW: 5'-CGC CGA GCG CAG AAG TGG TC-3' and RV: 5'-CTG CCG GCT GGC TGG TTT ATT G-3'. We constructed pJET-CpGFP and pJET-SumoBPTI in this manner.

The building block of the anionic ELP gene (E36) and neutral ELP gene (V40) on PUC19 was constructed previously by our group.[1] The recognition sites of the restriction enzymes PflMI and BglI were preserved by incorporating one valine instead of a glutamic acid residue per ten pentapeptide repeats. The ELP gene was cut from the PUC19 vector by digestion with PflMI and BglI and run on a 1% agarose gel in TAE buffer. The band containing the ELP gene was excised from the gel and purified using a spin column purification kit (General Electric). pJET with target fragments were also digested with PflMI and BglI and dephosphorylated with Fast AP. The vectors were purified by 1% agarose gel extraction. The linearized pJET vectors and the ELP-encoding gene were ligated using T4 ligase with a molar ratio 1:3 and transformed into chemically competent DH5α cells. Cells were plated and colonies were picked and grown in LB medium supplemented with 100 µg/mL carbenicillin overnight, and plasmids were isolated using the GeneJET Plasmid Miniprep kit. Positive clones were verified by analytical digest with PflMI and BglI following gel electrophoresis. The DNA sequences of the inserts were verified by DNA sequencing (Microsynth Sequencing AG). Gene oligomerization was performed as described by Chilkoti and co-workers.[2] Finally, the gene fragments encoding the ELP fusion proteins were transferred into the expression vector pET25b(+) for protein expression.

Protein expression and purification

E. coli BLR(DE3) cells (Novagen) were chosen as expression stains because this strain stabilizes plasmids with repetitive sequences.[3] For protein production, overnight culture was diluted 1/100 in TB medium supplemented with 100 µg/mL carbenicillin and incubated at 37°C shaking at 200 rpm until OD600 0.8-1.0. Protein production was initiated with 1 mM IPTG at 22°C for GFPs and 30 °C for BPTIs for at least 16h. Cells were subsequently harvested by centrifugation (6,000×g, 20 min, 4ºC), resuspended in lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.5 mM PMSF, 10 µg/ml DNaseI) and disrupted by high pressure homogenizer (Constant Systems Ltd Muti-Shot). Insoluble cell debris was removed by centrifugation (15,000×g, 30 min, 4 °C). Proteins were purified from the supernatant under native conditions by Ni-sepharose chromatography: Supernatant was filtered using a 0.22 µm pore size membrane filter (Millipore Corp.), and loaded onto a Histrap fast flow column (General Electric), pre-equilibrated with His-binding buffer (50 mM Phosphate Buffer, 500 mM NaCl, 20 mM Imidazole, pH=7.4). Next, 5-8 column volumes of His-washing buffer (50 mM Phosphate Buffer, 500 mM NaCl, 50 mM imidazole, pH=7.4) was added to remove impurities, after which 3 column volumes of His-elution buffer (50 mM phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH=7.4) was used to collect the target protein. The product was further purified by ion exchange chromatography (Q HP column for glutamic acid containing SUPs; herparin HP column for lysine containing SUPs). To this end, the product was 10 times diluted with 50 mM Phosphate Buffer (pH=8) and loaded onto a pre-equilibrated (IEC A buffer: 50 mM Phosphate Buffer, 20 mM NaCl, pH=8) ion exchange column. The supercharged target protein was purified with a 10 column volumes gradient elution program from 100% IEC A buffer to 100% IEC B (50 mM Phosphate Buffer, 2 M NaCl, pH=8) buffer with a flow speed of 0.5 ml/min. We collected the first fraction with supercharged GFP for our experiments, which gave good spectral properties. The concentrations of the purified ELP fusion proteins were determined by absorbance at 280 nm using a Microplate Reader Spectrophotometer (Molecular Devices SpectraMax M3). Protein purity was determined on a 15% SDS-PAGE stained with Coomassie staining solution (40% methanol, 10% glacial acetic acid, 1 g/L Brilliant Blue R250), and analyzed with ImageJ software. Photographs of the gels were taken with Bio-Rad gel imager (E-box, Vilber).

Protein Cleavage

To determine the influence of the SUMO-tag on the binding affinity between trypsin and BPTI and its role in protein sensitivity with ultrasound, SUMO protease (Sigma) was used to cleave the SUMO-tag. 20 units of SUMO protease was used per mg of target protein for 1 hour at 30 ºC or overnight at 2–8ºC. Products were purified by Ni-NTA. The reaction mixture was loaded onto a Histrap fast flow column (General Electric) pre-equilibrated with the His-binding buffer (50 mM phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH=7.4). 2-3 column volumes of His-binding buffer were used to collect BPTIs, which did not contain a His tag after reaction. The SUMO and SUMO protease have a His-tag and remain on the column.

Optical Spectroscopy

A Microplate Reader Spectrophotometer (Molecular Devices SpectraMax M3) was used for all absorbance and fluorescence measurements at room temperature in PBS buffer (pH=7.4), and protein concentrations were in the 10 µM range.

The CD spectra were obtained using a J-1500 CD Spectrometer (JASCO), and protein concentrations were in the 10 µM range. The following parameters were used: CD scale 200 mdeg/1.0 dOD, D.I.T. 2 sec, Bandwidth 1 nm.

Sonication

A QSonica sonicator (20 kHz) was used in this study. The 422-A probe (50% amplitude) was used for all sonication experiments. The program was a loop with 2 s “on” following 1 s “off”. Everything used for sonication was pre-cooled on ice and the 1.5 mL Eppendorf tubes containing 500 µL of the samples were placed in ice-water bathed steel container during sonication.

ITC measurements

ITC measurements were carried out on a MicroCal PEAQ-ITC (Malvern) at 25 °C. Protein samples were dissolved in PBS (pH = 7.4). Trypsin (Sigma) was then dissolved using the 1×PBS buffer. All solutions were degassed before titrations were performed. The sample cell was filled with 180 µL trypsin (10 µM), and the syringe was filled with 40 µL BPTI (100 µM). During each experiment, 0.4 µL BPTI was first injected into the sample cell, followed by 19 injections of 2 µL BPTI at intervals of 60 s. To subtract the heat of the dilution, the ITC buffer was injected into the protein solution under the same conditions. The raw titration data were analyzed using the device attached software. The calculated K_D value was the average of triplicate measurements.

Dynamic light scattering (DLS)

DLS (Fig. S2) was conducted with a Zetasizer Ultra (Malvern) operating with a Multi-Angle Dynamic Light Scattering method at 25 °C. Samples were filtered (0.22 µm pore size, Millipore Corp.) before analysis.

Enzymatic assay of trypsin

Trypsin activity was determined by using *N*α-Benzoyl-L-arginine ethyl ester (BAEE) as the substrate. BAEE can be digested into *N*α-Benzoyl-L-arginine, which has a specific absorbance at 253 nm. By recording the increase at this wavelength, the enzymatic kinetics were determined. For this purpose, 3.13 mL substrate solution with 0.23 mM BAEE and 0.031-0.063 mM hydrochloric acid in PBS buffer (pH = 7.6) were prepared and equilibrated to 25 °C. Then, 10 µL of 43 µM trypsin and 65 µL of 26.5 µM BPTI inhibitors were mixed and incubated for 10 min to form the deactivated trypsin/BPTI complex. To this solution, 425 µL substrate solution was added, and the mixture was subjected to sonication for a given time. After 10 s of lag time after sonication, the mixture (0.5 mL) was added to the residual 2.7 mL substrate solution to reach a final reaction volume of 3.2 mL. Finally, the absorbance at 253 nm of the reaction mixture was recorded for 10 min. From this measurement, the linear absorbance increase (ΔA_{253}/min) in the first minute period was obtained and further used to calculate the specific activity of trypsin.

Protein Sequences

GFP sequence used in this study was from Superfolder GFP.[4] The BPTI sequence used was from aprotinin.[5]

GFP-GS(GFP11-GS-GFP1-10)

MHHHHHHMRDHMVLHEYVNAAGITHGMDELYKGGGSGGGSHGRPGGGSGGGSMSKGEELFTGVVPILVELDGDVNGHKFSVRGE
GEGDATIGKLTLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGKYKTRAVVKFEGDTLV
NRIELKGTDFKEDGNILGHKLEYNFNSHNVYIMADKQKNGIKVNFTVRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQTVLSK
DPNEK

GFP-E36(GFP11-E36-GFP1-10)

MHHHHHHMRDHMVLHEYVNAAGITHGMDELYKGGGSGGGSHGVG[(VPGEG)$_9$(VPGVG)]$_3$(VPGEG)$_9$VPGRPGGGSGGGSMSKGE
ELFTGVVPILVELDGDVNGHKFSVRGEGEGDATIGKLTLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYV
QERTIFFKDDGKYKTRAVVKFEGDTLVNRIELKGTDFKEDGNILGHKLEYNFNSHNVYIMADKQKNGIKVNFTVRHNVEDGSVQLADHY
QQNTPIGDGPVLLPDNHYLSTQTVLSKDPNEK

GFP-E72(GFP11-E72-GFP1-10)

MHHHHHHMRDHMVLHEYVNAAGITHGMDELYKGGGSGGGSHGVG[(VPGEG)$_9$(VPGVG)]$_7$(VPGEG)$_9$VPGRPGGGSGGGSMSKGE
ELFTGVVPILVELDGDVNGHKFSVRGEGEGDATIGKLTLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYV
QERTIFFKDDGKYKTRAVVKFEGDTLVNRIELKGTDFKEDGNILGHKLEYNFNSHNVYIMADKQKNGIKVNFTVRHNVEDGSVQLADHY
QQNTPIGDGPVLLPDNHYLSTQTVLSKDPNEK

GFP-V40(GFP11-V40-GFP1-10)

MHHHHHHMRDHMVLHEYVNAAGITHGMDELYKGGGSGGGSHGVG(VPGVG)$_{39}$VPGRPGGGSGGGSMSKGEELFTGVVPILVELDG
DVNGHKFSVRGEGEGDATIGKLTLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGKYKT
RAVVKFEGDTLVNRIELKGTDFKEDGNILGHKLEYNFNSHNVYIMADKQKNGIKVNFTVRHNVEDGSVQLADHYQQNTPIGDGPVLLPD
NHYLSTQTVLSKDPNEK

Sumo-BPTI

MHHHHHHMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLD
MEDNDIIEAHREQIGGGGGSRPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGAGGGSHGVG
RKRGKWPGRP

Sumo-BPTI-K36

MHHHHHHMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLD
MEDNDIIEAHREQIGGGGGSRPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGAGGGSHGVG[(
VPGKG)$_9$(VPGVG)]$_3$(VPGKG)$_9$VPGRP

DNA sequence

GFP-GS(GFP11-GS-GFP1-10)

ATGCACCATCACCACCACCACATGCGCGATCACATGGTACTTCACGAATACGTCAACGCAGCAGGTATCACTCACGGGATGGAC
GAACTGTATAAAGGAGGTGGAAGCGGAGGCGGTTCCCACGGCCGGCCAGGGGGAGGTTCAGGTGGCGGTTCTATGTCCAAAG
GGGAAGAGTTATTCACTGGTGTTGTGCCGATCTTGGTTGAGCTTGATGGTGATGTAAACGGTCATAAATTTTCCGTGCGCGGTGA
AGGCGAGGGTGACGCGACTATCGGAAAATTGACCTTGAAGTTCATTTGCACCACGGGAAAGTTACCCGTCCCTTGGCCCACATT
AGTGACGACATTTAGCTACGGGGTCCAGTGTTTTTCGCGCTACCCGGACCACATGAAGCAGCACGATTTCTTCAAGTCAGCGAT
GCCCGAGGGCTATGTACAGGAGCGTACTATCTTCTTTAAGGACGACGGCAAGTATAAGACTCGTGCAGTTGTAAAATTTGAGGG
GGATACCTTGGTCAATCGTATCGAGCTTAAAGGTACCGATTTTAAAGAAGATGGAAATATTCTTGGACACAAACTTGAATACAACT
TTAACTCCCATAATGTATATATCATGGCTGATAAACAGAAAAACGGGATCAAAGTCAACTTCACGGTACGTCACAACGTAGAGGAT
GGTTCCGTTCAGTTAGCGGACCATTATCAACAGAATACTCCCATTGGTGATGGCCCCGTTCTGTTACCTGACAACCACTATCTGT
CAACTCAGACAGTATTGTCCAAGGACCCGAATGAGAAA

GFP-E36(GFP11-E36-GFP1-10)

ATGCACCATCACCACCACCACATGCGCGATCACATGGTACTTCACGAATACGTCAACGCAGCAGGTATCACTCACGGGATGGAC
GAACTGTATAAAGGAGGTGGAAGCGGAGGCGGTTCCCACGGCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGC
CAGGCGAAGGTGTTCCGGGTGAAGGTGTGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGA
AGGCGTACCAGGTGAAGGTGTGCCGGGCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGCCAGGCGAAGGTGTT
CCGGGTGAAGGTGTGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGAAGGCGTACCAGGT
GAAGGTGTGCCGGGCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGCCAGGCGAAGGTGTTCCGGGTGAAGGTG
TGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGAAGGCGTACCAGGTGAAGGTGTGCCGG
GCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGCCAGGCGAAGGTGTTCCGGGTGAAGGTGTGCCGGGTGAAGG
CGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGAAGGCGTACCAGGTGAAGGTGTGCCGGGCCGGCCAGGGGG
AGGTTCAGGTGGCGGTTCTATGTCCAAAGGGGAAGAGTTATTCACTGGTGTTGTGCCGATCTTGGTTGAGCTTGATGGTGATGT
AAACGGTCATAAATTTTCCGTGCGCGGTGAAGGCGAGGGTGACGCGACTATCGGAAAATTGACCTTGAAGTTCATTTGCACCAC
GGGAAAGTTACCCGTCCCTTGGCCCACATTAGTGACGACATTTAGCTACGGGGTCCAGTGTTTTTCGCGCTACCCGGACCACAT
GAAGCAGCACGATTTCTTCAAGTCAGCGATGCCCGAGGGCTATGTACAGGAGCGTACTATCTTCTTTAAGGACGACGGCAAGTA
TAAGACTCGTGCAGTTGTAAAATTTGAGGGGGATACCTTGGTCAATCGTATCGAGCTTAAAGGTACCGATTTTAAAGAAGATGGA
AATATTCTTGGACACAAACTTGAATACAACTTTAACTCCCATAATGTATATATCATGGCTGATAAACAGAAAAACGGGATCAAAGTC
AACTTCACGGTACGTCACAACGTAGAGGATGGTTCCGTTCAGTTAGCGGACCATTATCAACAGAATACTCCCATTGGTGATGGCC
CCGTTCTGTTACCTGACAACCACTATCTGTCAACTCAGACAGTATTGTCCAAGGACCCGAATGAGAAA

GFP-E72(GFP11-E72-GFP1-10)

ATGCACCATCACCACCACCACATGCGCGATCACATGGTACTTCACGAATACGTCAACGCAGCAGGTATCACTCACGGGATGGAC
GAACTGTATAAAGGAGGTGGAAGCGGAGGCGGTTCCCACGGCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGC
CAGGCGAAGGTGTTCCGGGTGAAGGTGTGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGA
AGGCGTACCAGGTGAAGGTGTGCCGGGCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGCCAGGCGAAGGTGTT
CCGGGTGAAGGTGTGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGAAGGCGTACCAGGT
GAAGGTGTGCCGGGCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGCCAGGCGAAGGTGTTCCGGGTGAAGGTG
TGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGAAGGCGTACCAGGTGAAGGTGTGCCGG
GCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGCCAGGCGAAGGTGTTCCGGGTGAAGGTGTGCCGGGTGAAGG
CGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGAAGGCGTACCAGGTGAAGGTGTGCCGGGCGTGGGTGTTCCG
GGTGAAGGTGTTCCGGGCGAAGGTGTGCCAGGCGAAGGTGTTCCGGGTGAAGGTGTGCCGGGTGAAGGCGTACCGGGTGAAG
GCGTACCAGGCGAAGGTGTTCCGGGTGAAGGCGTACCAGGTGAAGGTGTGCCGGGCGTGGGTGTTCCGGGTGAAGGTGTTCC
GGGCGAAGGTGTGCCAGGCGAAGGTGTTCCGGGTGAAGGTGTGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGA
AGGTGTTCCGGGTGAAGGCGTACCAGGTGAAGGTGTGCCGGGCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTG

CCAGGCGAAGGTGTTCCGGGTGAAGGTGTGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTG
AAGGCGTACCAGGTGAAGGTGTGCCGGGCGTGGGTGTTCCGGGTGAAGGTGTTCCGGGCGAAGGTGTGCCAGGCGAAGGTGT
TCCGGGTGAAGGTGTGCCGGGTGAAGGCGTACCGGGTGAAGGCGTACCAGGCGAAGGTGTTCCGGGTGAAGGCGTACCAGGT
GAAGGTGTGCCGGGCCGGCCAGGGGGAGGTTCAGGTGGCGGTTCTATGTCCAAAGGGGAAGAGTTATTCACTGGTGTTGTGCC
GATCTTGGTTGAGCTTGATGGTGATGTAAACGGTCATAAATTTTCCGTGCGCGGTGAAGGCGAGGGTGACGCGACTATCGGAAA
ATTGACCTTGAAGTTCATTTGCACCACGGGAAAGTTACCCGTCCCTTGGCCCACATTAGTGACGACATTTAGCTACGGGGTCCAG
TGTTTTTCGCGCTACCCGGACCACATGAAGCAGCACGATTTCTTCAAGTCAGCGATGCCCGAGGGCTATGTACAGGAGCGTACT
ATCTTCTTTAAGGACGACGGCAAGTATAAGACTCGTGCAGTTGTAAAATTTGAGGGGGATACCTTGGTCAATCGTATCGAGCTTA
AAGGTACCGATTTTAAAGAAGATGGAAATATTCTTGGACACAAACTTGAATACAACTTTAACTCCCATAATGTATATATCATGGCTG
ATAAACAGAAAAACGGGATCAAAGTCAACTTCACGGTACGTCACAACGTAGAGGATGGTTCCGTTCAGTTAGCGGACCATTATCA
ACAGAATACTCCCATTGGTGATGGCCCCGTTCTGTTACCTGACAACCACTATCTGTCAACTCAGACAGTATTGTCCAAGGACCCG
AATGAGAAA

GFP-V40(GFP11-V40-GFP1-10)

ATGCACCATCACCACCACCACATGCGCGATCACATGGTACTTCACGAATACGTCAACGCAGCAGGTATCACTCACGGGATGGAC
GAACTGTATAAAGGAGGTGGAAGCGGAGGCGGTTCCCACGGCGTGGGTGTTCCGGGTGTCGGTGTTCCGGGCGTCGGTGTGC
CAGGCGTCGGTGTTCCGGGTGTCGGTGTGCCGGGTGTCGGCGTACCGGGTGTCGGCGTACCAGGCGTCGGTGTTCCGGGTGT
CGGCGTACCAGGTGTCGGTGTGCCGGGCGTGGGTGTTCCGGGTGTCGGTGTTCCGGGCGTCGGTGTGCCAGGCGTCGGTGTT
CCGGGTGTCGGTGTGCCGGGTGTCGGCGTACCGGGTGTCGGCGTACCAGGCGTCGGTGTTCCGGGTGTCGGCGTACCAGGT
GTCGGTGTGCCGGGCGTGGGTGTTCCGGGTGTCGGTGTTCCGGGCGTCGGTGTGCCAGGCGTCGGTGTTCCGGGTGTCGGTG
TGCCGGGTGTCGGCGTACCGGGTGTCGGCGTACCAGGCGTCGGTGTTCCGGGTGTCGGCGTACCAGGTGTCGGTGTGCCGG
GCGTGGGTGTTCCGGGTGTCGGTGTTCCGGGCGTCGGTGTGCCAGGCGTCGGTGTTCCGGGTGTCGGTGTGCCGGGTGTCGG
CGTACCGGGTGTCGGCGTACCAGGCGTCGGTGTTCCGGGTGTCGGCGTACCAGGTGTCGGTGTGCCGGGCCGGCCAGGGGG
AGGTTCAGGTGGCGGTTCTATGTCCAAAGGGGAAGAGTTATTCACTGGTGTTGTGCCGATCTTGGTTGAGCTTGATGGTGATGT
AAACGGTCATAAATTTTCCGTGCGCGGTGAAGGCGAGGGTGACGCGACTATCGGAAAATTGACCTTGAAGTTCATTTGCACCAC
GGGAAAGTTACCCGTCCCTTGGCCCACATTAGTGACGACATTTAGCTACGGGGTCCAGTGTTTTTCGCGCTACCCGGACCACAT
GAAGCAGCACGATTTCTTCAAGTCAGCGATGCCCGAGGGCTATGTACAGGAGCGTACTATCTTCTTTAAGGACGACGGCAAGTA
TAAGACTCGTGCAGTTGTAAAATTTGAGGGGGATACCTTGGTCAATCGTATCGAGCTTAAAGGTACCGATTTTAAAGAAGATGGA
AATATTCTTGGACACAAACTTGAATACAACTTTAACTCCCATAATGTATATATCATGGCTGATAAACAGAAAAACGGGATCAAAGTC
AACTTCACGGTACGTCACAACGTAGAGGATGGTTCCGTTCAGTTAGCGGACCATTATCAACAGAATACTCCCATTGGTGATGGCC
CCGTTCTGTTACCTGACAACCACTATCTGTCAACTCAGACAGTATTGTCCAAGGACCCGAATGAGAAA

Sumo-BPTI

ATGCACCATCATCATCATCATATGTCGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCTGAGA
CTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGATGGAA
GCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGATCAGACCCCTG
AAGATTTGGACATGGAGGATAACGATATTATTGAGGCTCACAGAGAACAGATTGGTGGTGGAGGTGGAAGCCGTCCGGATTTTT
GCCTGGAACCGCCATATACCGGTCCGTGTAAAGCACGCATTATCCGCTACTTCTATAACGCGAAAGCCGGTCTGTGCCAGACGT
TTGTGTATGGCGGTTGCCGTGCGAAGCGCAATAACTTCAAAAGCGCGGAAGATTGTATGCGTACCTGCGGTGGCGCAGGAGGT
GGAAGCCACGGCGTGGGGAGGAAGCGGGGGAAGTGGCCAGGCCGGCCA

Sumo-BPTI-K36

ATGCACCATCATCATCATCATATGTCGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCTGAGA
CTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGATGGAA
GCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGATCAGACCCCTG
AAGATTTGGACATGGAGGATAACGATATTATTGAGGCTCACAGAGAACAGATTGGTGGTGGAGGTGGAAGCCGTCCGGATTTTT
GCCTGGAACCGCCATATACCGGTCCGTGTAAAGCACGCATTATCCGCTACTTCTATAACGCGAAAGCCGGTCTGTGCCAGACGT
TTGTGTATGGCGGTTGCCGTGCGAAGCGCAATAACTTCAAAAGCGCGGAAGATTGTATGCGTACCTGCGGTGGCGCAGGAGGT
GGAAGCCACGGCGTGGGTGTTCCGGGTAAAGGTGTTCCGGGCAAAGGTGTGCCAGGCAAAGGTGTTCCGGGTAAAGGTGTGC
CGGGTAAAGGCGTACCGGGTAAAGGCGTACCAGGCAAAGGTGTTCCGGGTAAAGGCGTACCAGGTAAAGGTGTGCCGGGCGT

GGGTGTTCCGGGTAAAGGTGTTCCGGGCAAAGGTGTGCCAGGCAAAGGTGTTCCGGGTAAAGGTGTGCCGGGTAAAGGCGTA
CCGGGTAAAGGCGTACCAGGCAAAGGTGTTCCGGGTAAAGGCGTACCAGGTAAAGGTGTGCCGGGCGTGGGTGTTCCGGGTA
AAGGTGTTCCGGGCAAAGGTGTGCCAGGCAAAGGTGTTCCGGGTAAAGGTGTGCCGGGTAAAGGCGTACCGGGTAAAGGCGT
ACCAGGCAAAGGTGTTCCGGGTAAAGGCGTACCAGGTAAAGGTGTGCCGGGCGTGGGTGTTCCGGGTAAAGGTGTTCCGGGC
AAAGGTGTGCCAGGCAAAGGTGTTCCGGGTAAAGGTGTGCCGGGTAAAGGCGTACCGGGTAAAGGCGTACCAGGCAAAGGTGT
TCCGGGTAAAGGCGTACCAGGTAAAGGTGTGCCGGGCCGGCCA

Results and Discussion

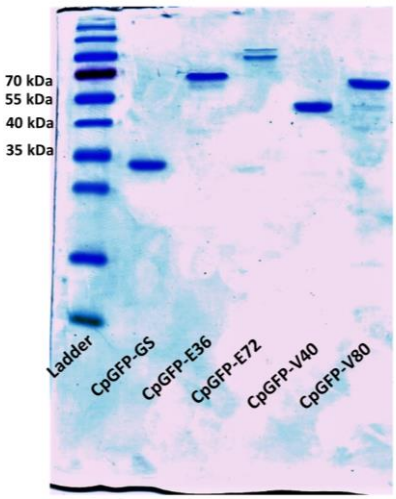


Figure S1. Coomassie-stained 15% SDS-PAGE characterization of GFPs used in this study. The mobility varies between similar molecular weights due to the highly charged nature of the ELPs.

Table S1. Expected molar mass of different GFP variants.

	GFP-GS	GFP-E36	GFP-E72	GFP-V40
Expected molar mass (Da)	29467.05	46925.86	64384.67	45846.48

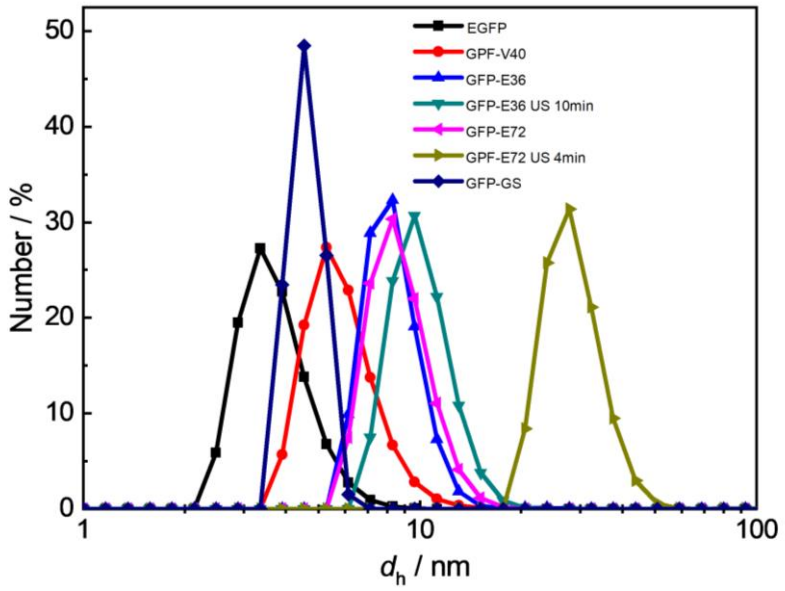


Figure S2. DLS analysis of different variants of GFP, where d_h is hydrodynamic diameter. All the protein samples were purified by size exclusion chromatography (Superdex 200 10/300 GL) before DLS measurements to remove aggregates. Buffer was exchanged to PBS buffer (pH = 7.4) during SEC and protein concentrations were in the 10 μM range.

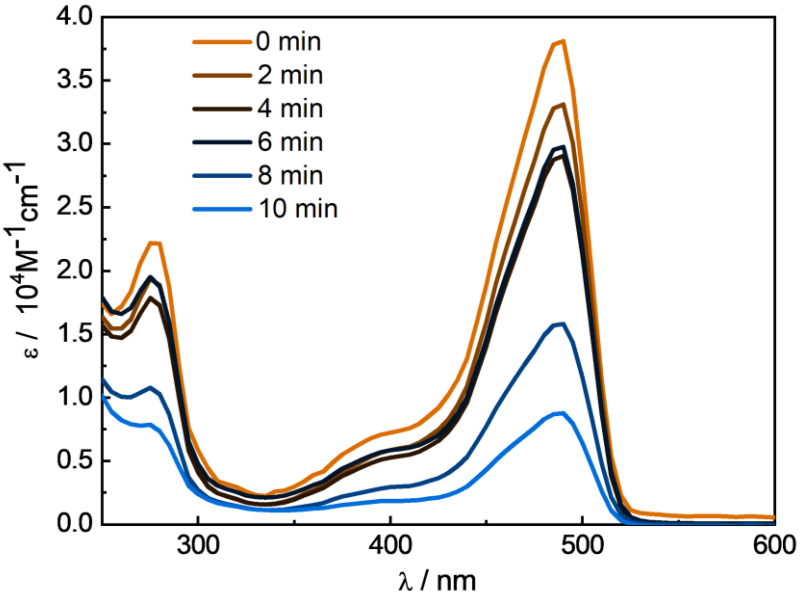


Figure S3. UV-vis absorption spectra of EGFP for different sonication times. The decrease in absorption at 280 nm and 470 nm indicates that ultrasound reduces the concentration of soluble EGFP. The protein sample used in the absorbance spectra was placed in PBS buffer (pH = 7.4) at a concentration of 10 μM. All samples were centrifuged to remove precipitates formed during sonication, and supernatants were used for the measurements. The spectra were obtained at room temperature.

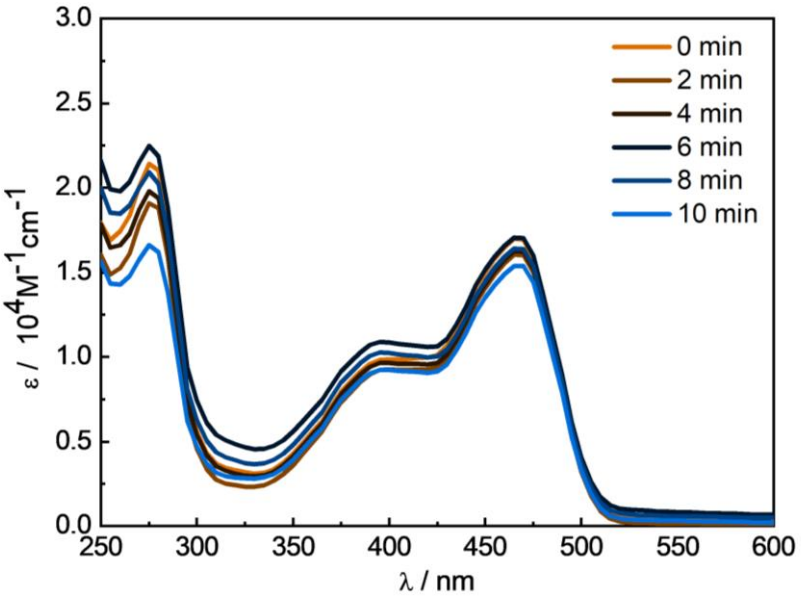


Figure S4. UV-vis absorption spectra of GFP-GS for different sonication times, showing relative little change. Samples were processed similar as EGFP in Fig. S3, and spectra were obtained in PBS buffer (pH = 7.4) at room temperature.

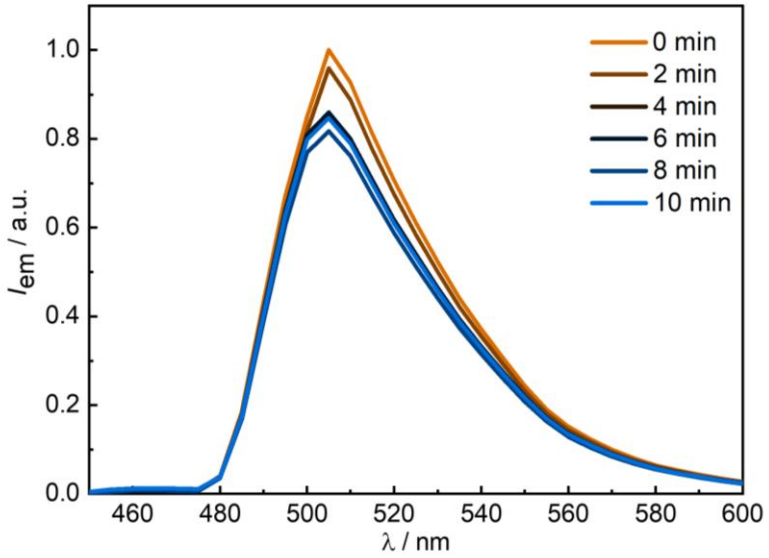


Figure S5. Fluorescence emission spectra of GFP-GS for different sonication times. GFP-GS showed more than 85% fluorescence after 10 min continuous sonication, suggesting that GFP-GS exhibits a relatively stable scaffold against ultrasound. Spectra were obtained with excitation at 468 nm in PBS buffer (pH = 7.4) at room temperature and normalized against the fluorescence intensity at 0 min.

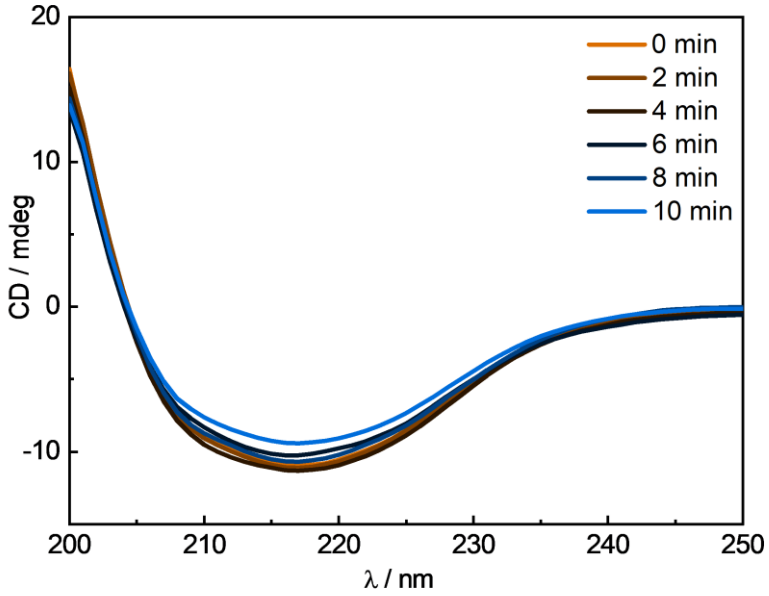
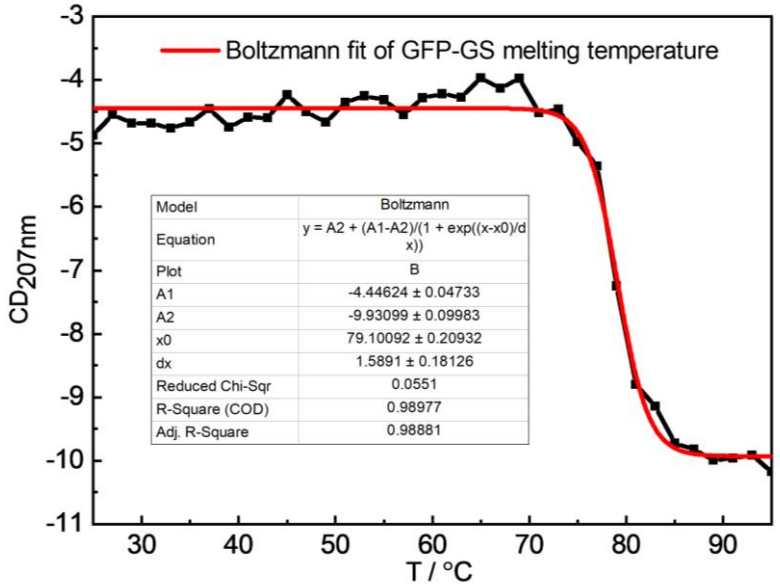


Figure S6. CD spectra of GFP-GS for different sonication times. GFP-GS retained its secondary structure after 10 min of continuous sonication. Spectra were obtained in PBS buffer (pH = 7.4) at room temperature with a HT voltage lower than 500 V.



Model	Boltzmann
Equation	y = A2 + (A1-A2)/(1 + exp((x-x0)/dx))
Plot	B
A1	-4.44624 ± 0.04733
A2	-9.93099 ± 0.09983
x0	79.10092 ± 0.20932
dx	1.5891 ± 0.18126
Reduced Chi-Sqr	0.0551
R-Square (COD)	0.98977
Adj. R-Square	0.98881

Figure S7. GFP-GS melting temperature determination. The melting temperature of GFP-GS was 78.6 ± 0.5 °C, slightly higher than the EGPF melting temperature of 76 °C. Variable-temperature circular dichroism was utilized to analyze GFP-GS melting temperature, and spectra were obtained in PBS buffer (pH = 7.4) at room temperature with a HT voltage lower than 500 V. The CD signal at 207 nm to be the y-axis and plotted versus the temperate. The melting temperature was determined employing a Boltzmann fit in Origin software.

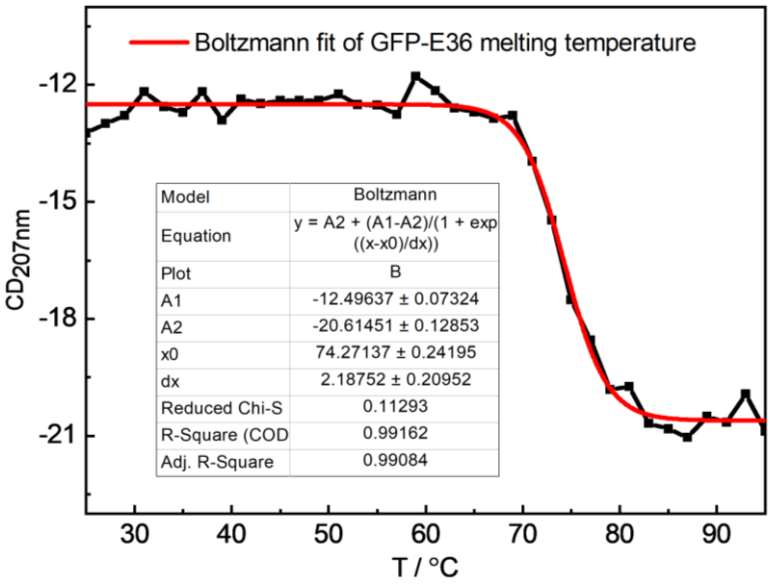


Figure S8. GFP-E36 melting temperature determination. The melting temperature of GFP-E36 was 74.3 ± 0.8 °C, a little lower than the EGPF melting temperature of 76 °C published before. Method and conditions as Fig. S7.

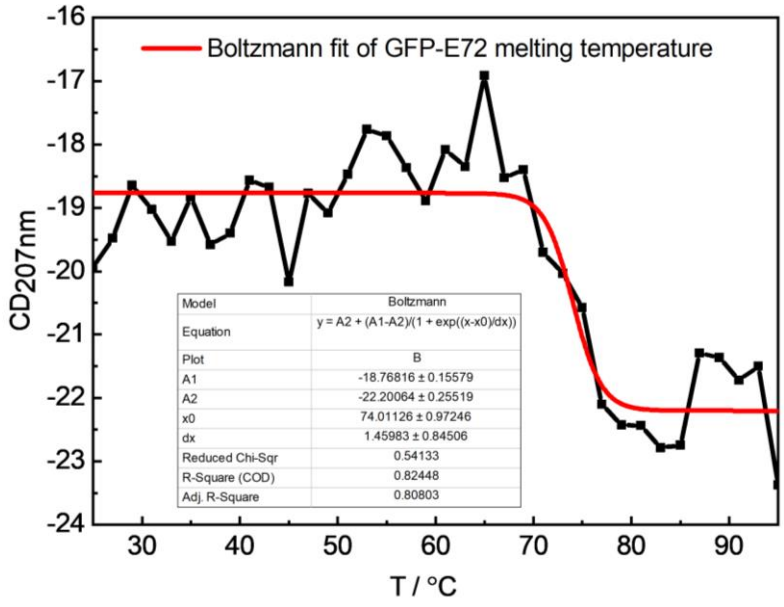


Figure S9. GFP-E72 melting temperature determination. The melting temperature of GFP-E72 was 73.4 ± 0.6 °C, a little lower than the GPF melting temperature of 76 °C published before. Method and conditions as Fig. S7.

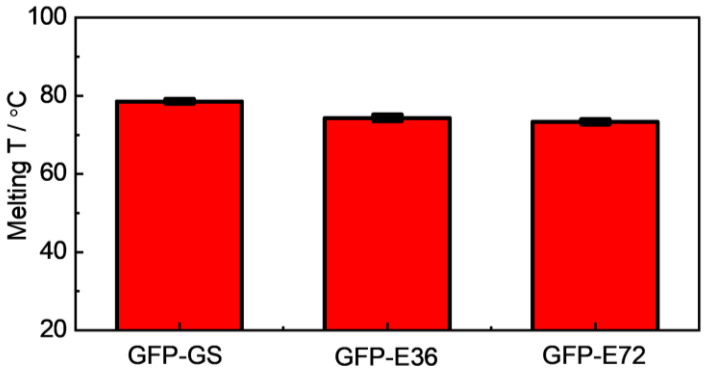


Figure S10. Summary of melting temperatures of GFP-GS, GFP-E36, and GFP-E72 as measured by variable-temperature circular dichroism. All experiments were repeated as triplicates and error bars represent standard deviations.

Table S2. Melting temperatures of different GFP variants.

Melting temperature (°C)	1	2	3	Mean value	SD from the mean
GFP-GS	79.1	78.6	78.1	78.6	0.5
GPF-E36	74.3	75.1	73.6	74.3	0.8
GFP-E72	74.0	73.2	72.9	73.4	0.6

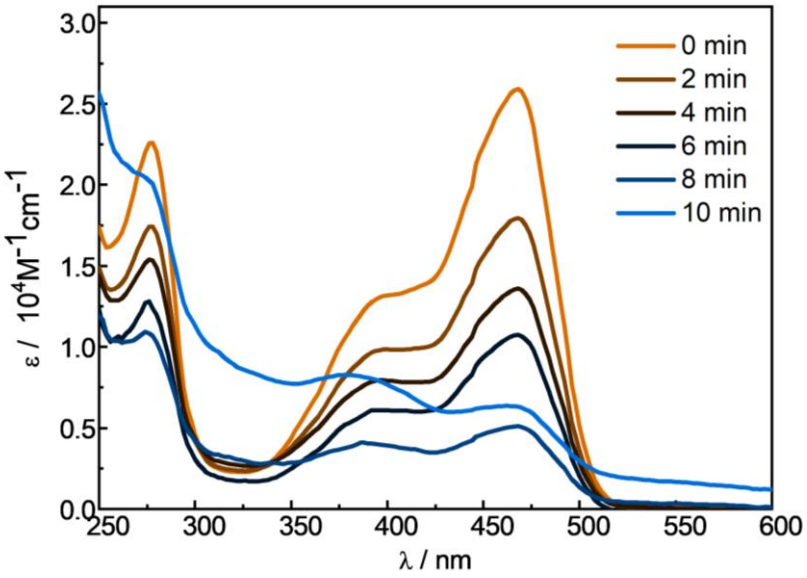


Figure S11. UV-vis absorption spectra of GFP-V40 for different sonication times. During sonication GFP-V40 precipitated from solution, similar to EGFP.

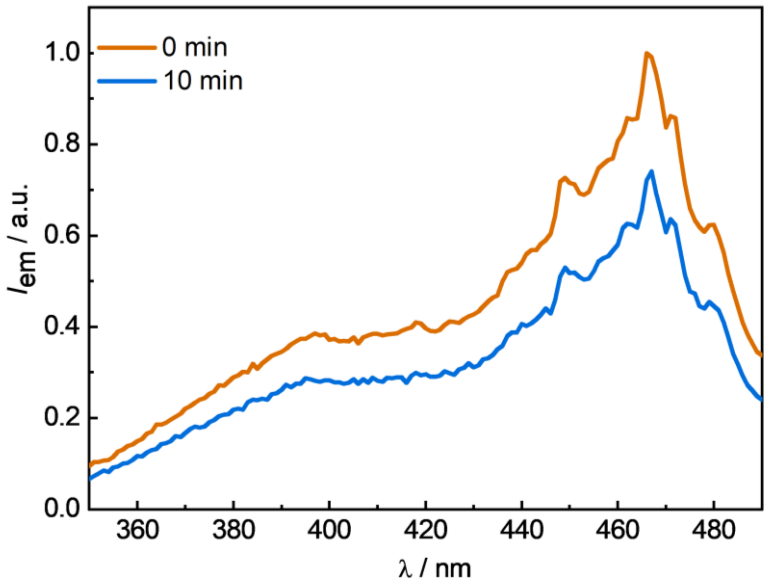


Figure S12. Fluorescence excitation spectra of GFP-E36 for different sonication times. The ratio between the bands at 400 nm and 470 nm did not change after 10 min sonication, suggesting that the ratio between protonated and deprotonated chromophores was not affected by sonication. Spectra were obtained with emission at 505 nm in PBS buffer (pH = 7.4) at room temperature and normalized to intensity at 0 min.

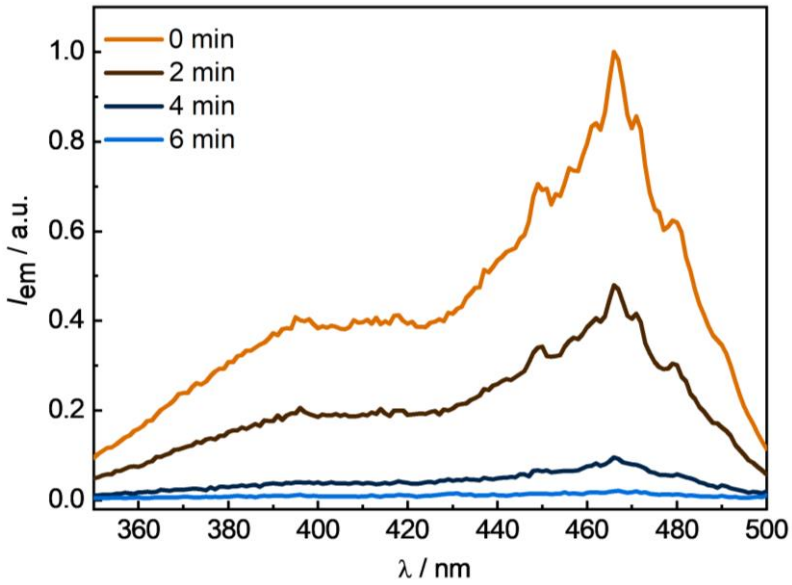


Figure S13. Fluorescence excitation spectra of GFP-E72 for different sonication times. Spectra were obtained with emission at 505 nm in PBS buffer (pH = 7.4) at room temperature and normalized to intensity at 0 min.

Table S3. Fluorescence quantum yields of different GFP variants.

	EGFP	GFP-GS	GFP-E36	GFP-E72	GFP-V40
Fluorescence quantum yield[a]	0.60	0.44	0.47	0.33	0.34

[a] Fluorescence quantum yield was calculated with $Y = Y_{ref} \times \frac{F_{ref}}{F} \times \frac{A}{A_{ref}}$, in which

Y = Fluorescence quantum yield of the protein of interest, Y_{ref} = Fluorescence quantum yield of reference protein (GFP), F = Intergrated fluorescence, F_{ref} = Reference integrated fluorescence, A = Absorbance, A_{ref} = Reference absorbance.

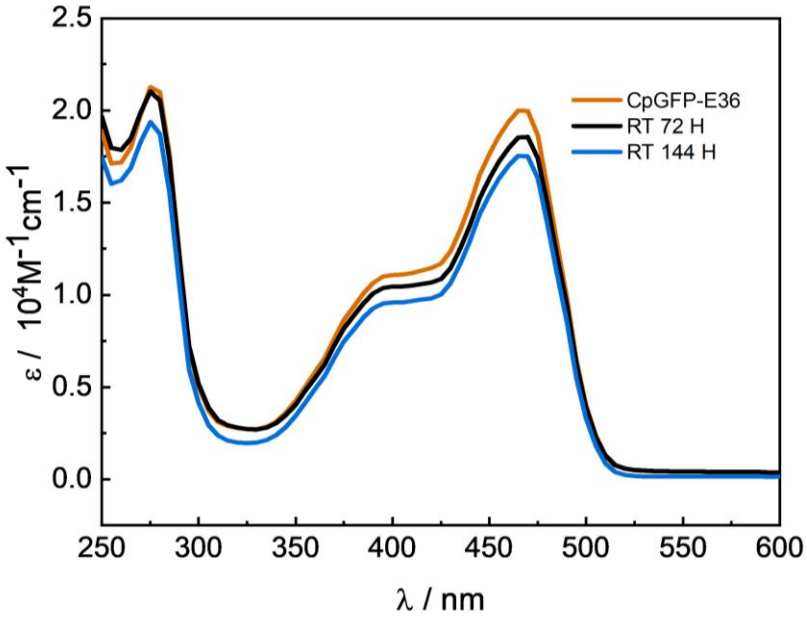


Figure S14. UV-vis absorption spectra of GFP-E36 incubated at room temperature for up to 144 h.

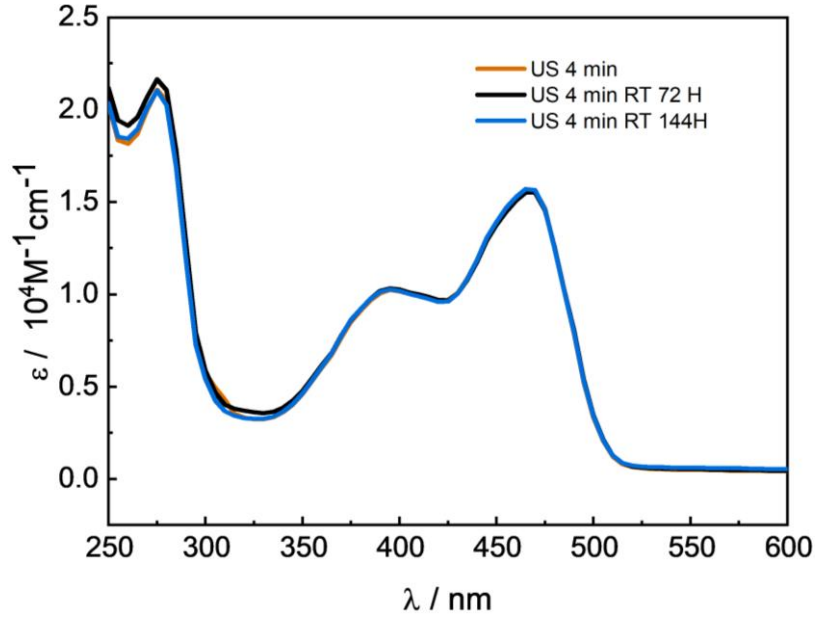


Figure S15. UV-vis absorption recovery spectra of GFP-E36 after 4 min ultrasonication and incubation at room temperature.

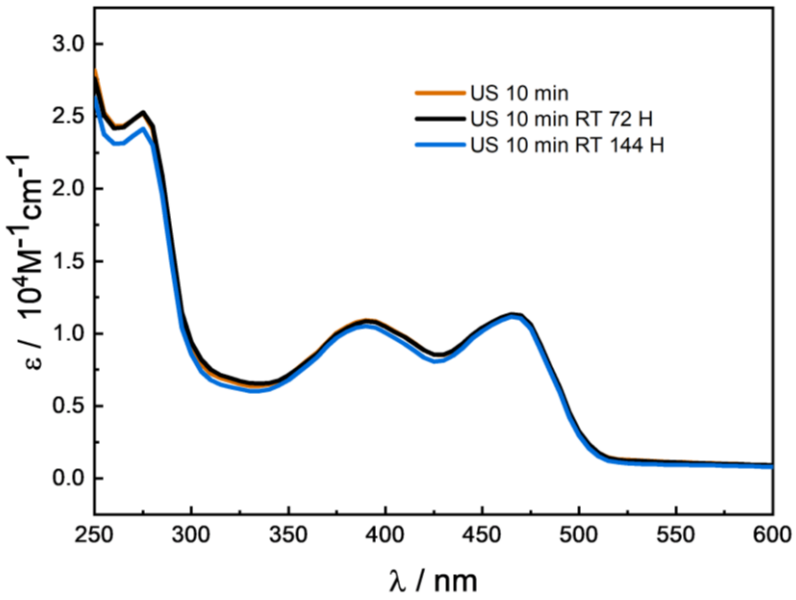


Figure S16. UV-vis absorption recovery spectra of GFP-E36 after 10 min ultrasonication and incubation at room temperature.

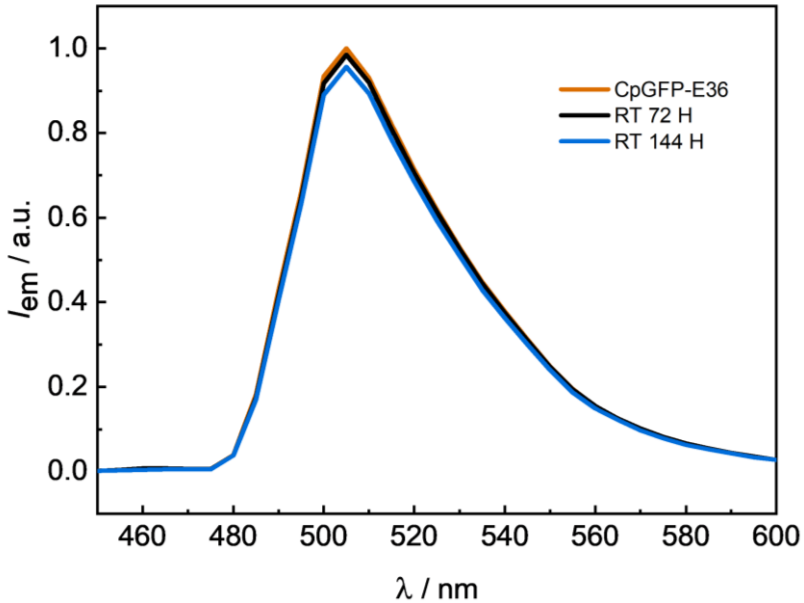


Figure S17. Fluorescence emission spectra of GFP-E36 incubated at room temperature. Spectra were obtained with excitation at 468 nm in PBS buffer (pH = 7.4) at room temperature and normalized against the fluorescence intensity of 10 μM GFP-E36 without sonication.

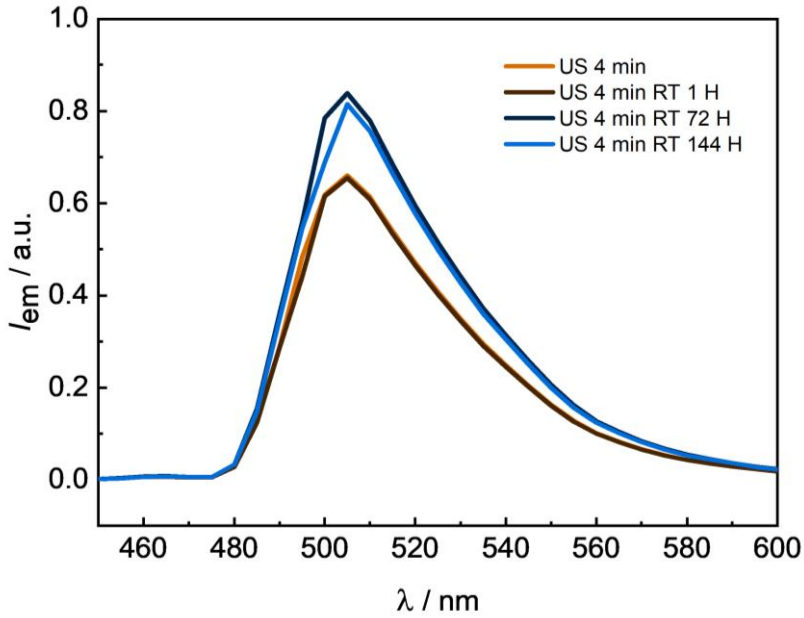


Figure S18. Fluorescence emission spectra of GFP-E36 after 4 min sonication and subsequently incubated at room temperature. Spectra were obtained with excitation at 468 nm in PBS buffer (pH = 7.4) at room temperature and normalized against the fluorescence intensity of 10 μM GFP-E36 without sonication.

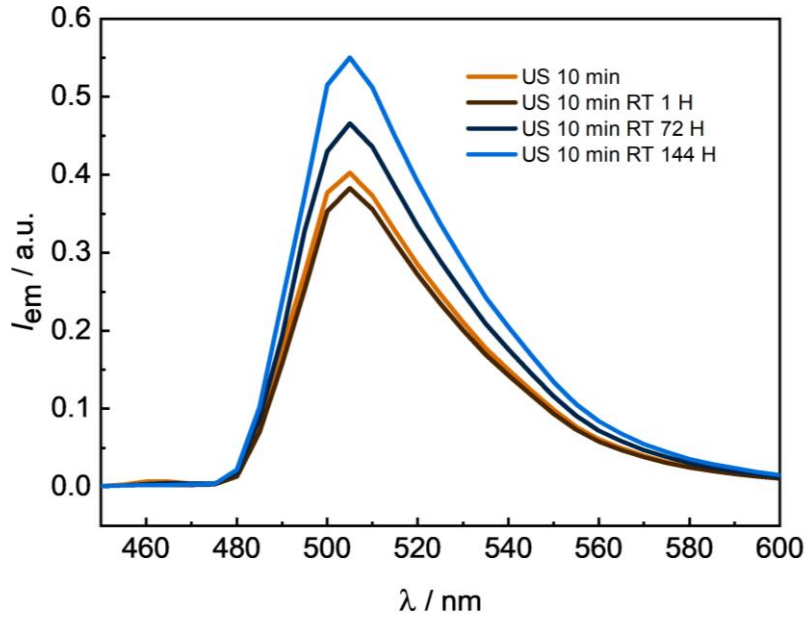


Figure S19. Fluorescence emission spectra of GFP-E36 after 10 min sonication and incubated at room temperature. Spectra were obtained with excitation at 468 nm in PBS buffer (pH = 7.4) at room temperature and normalized against the fluorescence intensity of 10 μM GFP-E36 without sonication.

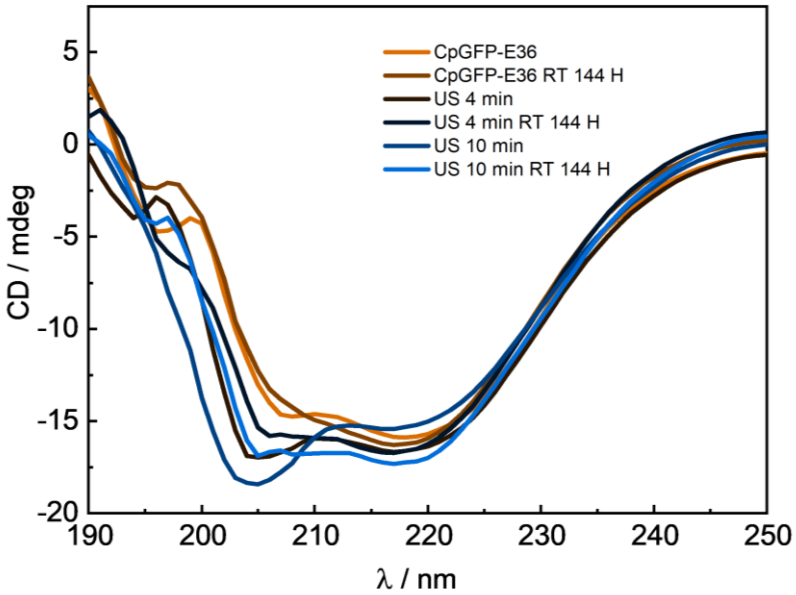


Figure S20. CD recovery spectra of GFP-E36 after ultrasonication and incubated at room temperature. GFP-E36 recovered part of its secondary structure after 144 h incubation. Spectra were obtained in PBS buffer (pH = 7.4) at room temperature with a HT voltage lower than 500 V.

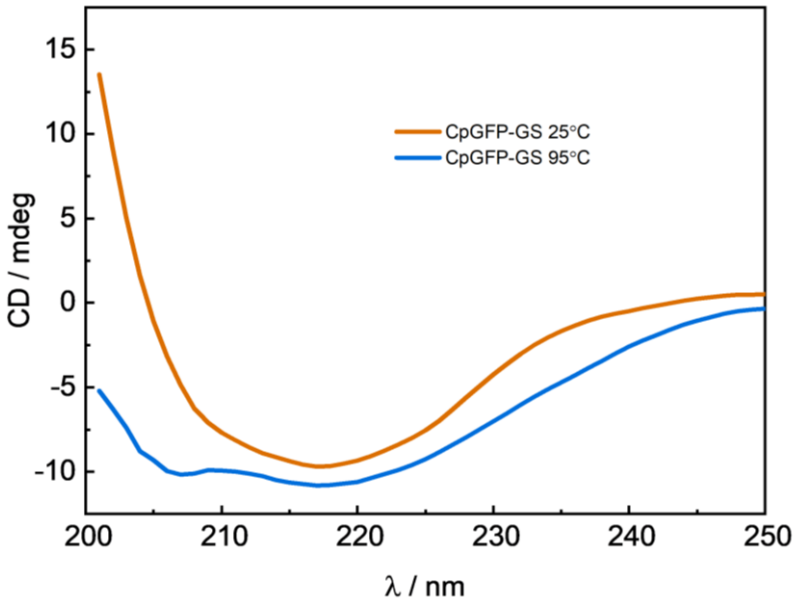


Figure S21. CD spectra of GFP-GS at 25 °C and 95 °C. Spectra were obtained in PBS buffer (pH = 7.4) at room temperature with a HT voltage lower than 500 V.

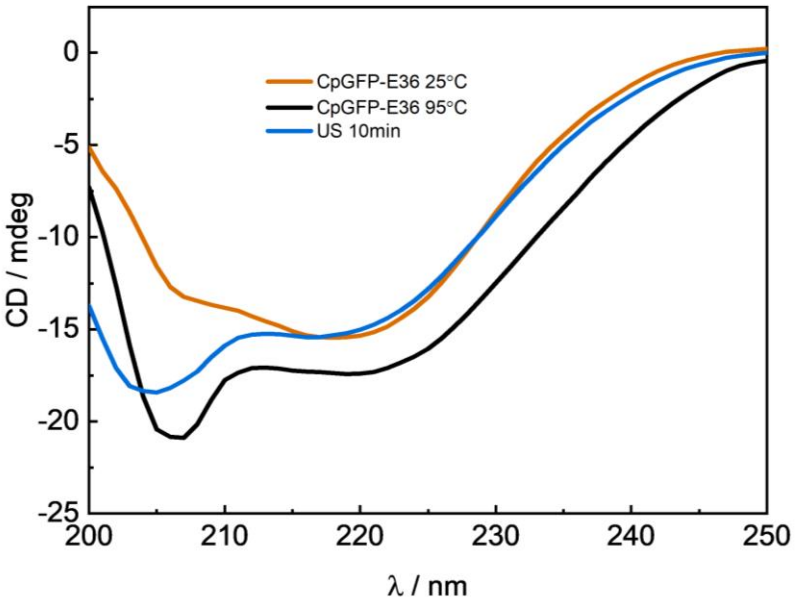


Figure S22. CD spectra of GFP-E36 at 25 °C, 95 °C, and after 10 min sonication. Sonication induced unfolding is different from temperature. Spectra were obtained in PBS buffer (pH = 7.4) at room temperature with a HT voltage lower than 500 V.

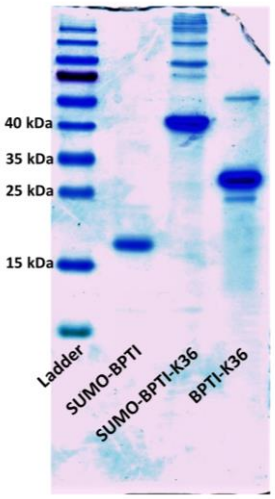


Figure S23. Coomassie-stained 15% SDS-PAGE characterization of BPTIs used in this work. The bands above the major product are most likely dimers or oligomeric forms of BPTI.

Table S4. Expected molar mass of different BPTI variants.

	SUMO-BPTI	SUMO-BPTI-K36	BPTI-K36
Expected molecular weight(Da)	19679.21	37104.13	24906.42

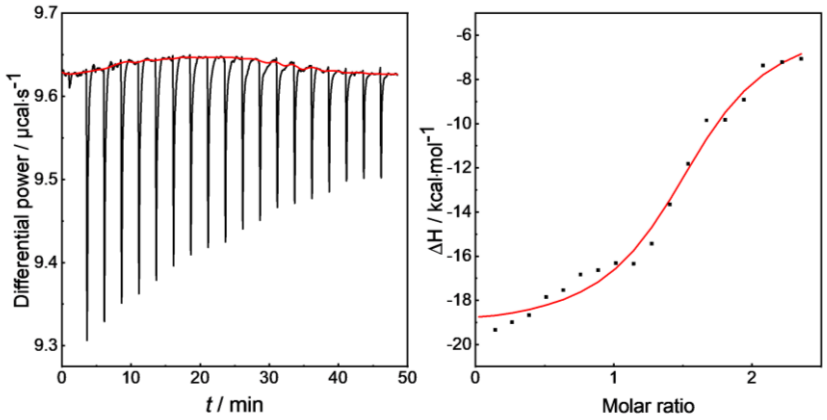


Figure S24. ITC characterization of SUMO-BPTI-K36 binding affinity with trypsin. The binding constant KD between SUMO-BPTI-K36 and trypsin is 5.44±0.39×10^{-7} M. Results were obtained in PBS buffer (pH = 7.4), the trypsin concentration in the bath was 8 μM, and the SUMO-BPTI-K36 concentration in the syringe was 100 μM. All experiments were repeated as triplicates.

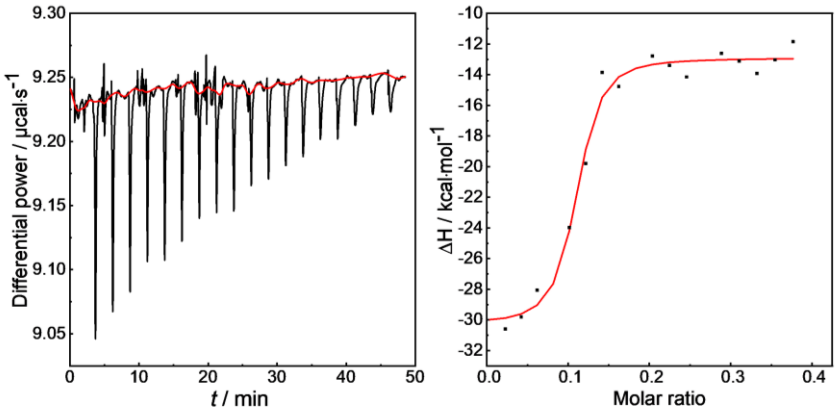


Figure S25. ITC characterization of BPTI-K36 binding affinity with trypsin. The binding constant KD between BPTI-K36 and trypsin is 4.53±0.42×10^{-8} M. Results were obtained in PBS buffer (pH = 7.4), the trypsin concentration in the bath was 20 μM and the BPTI-K36 concentration in the syringe was 40 μM. All experiments were repeated as triplicates.

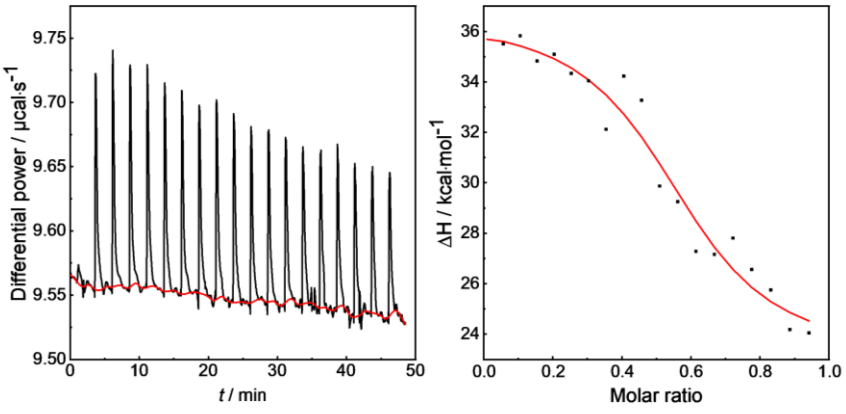


Figure S26. ITC characterization of SUMO-BPTI binding affinity with trypsin. The binding constant KD between SUMO-BPTI and trypsin is 3.70±0.53×10^{-7} M. Results were obtained in PBS buffer (pH = 7.4), the trypsin concentration in the bath was 8 μM and the BPTI-K36 concentration in the syringe was 100 μM. All experiments were repeated as triplicates.

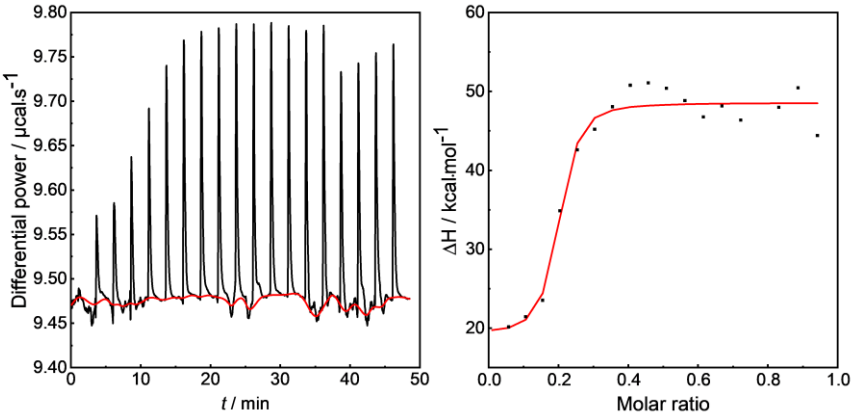


Figure S27. ITC characterization of BPTI binding affinity with trypsin. The binding constant K_D between BPTI and trypsin is 4.14 ± 0.13×10^{-8} M. Results were obtained in PBS buffer (pH = 7.4), the trypsin concentration in the bath was 10 µM, and the BPTI concentration in the syringe was 50 µM. All experiments were repeated as triplicates.

Table S5. Dissociation constants (K_D) of different BPTI variants.

Dissociation constant K_D (M)	1	2	3	Mean value	SD from the mean
SUMO-BPTI	3.75×10^{-7}	3.15×10^{-7}	4.2×10^{-7}	3.70×10^{-7}	0.53×10^{-7}
BPTI	4.03×10^{-8}	4.1×10^{-8}	4.28×10^{-8}	4.14×10^{-8}	0.13×10^{-8}
SUMO-BPTI-K36	5.35×10^{-7}	5.87×10^{-7}	5.1×10^{-7}	5.44×10^{-7}	0.39×10^{-7}
BPTI-K36	4.13×10^{-8}	4.5×10^{-8}	4.97×10^{-8}	4.53×10^{-8}	0.42×10^{-8}

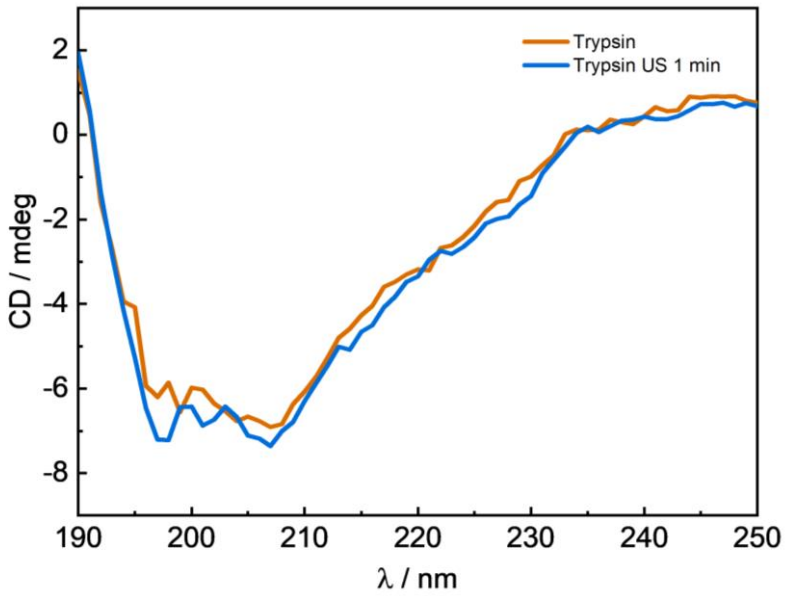


Figure S28. CD spectra of trypsin after 1 min sonication. Spectra were obtained using 0.5 mg/mL trypsin in PBS buffer (pH = 7.4) at room temperature with a HT voltage lower than 500 V.

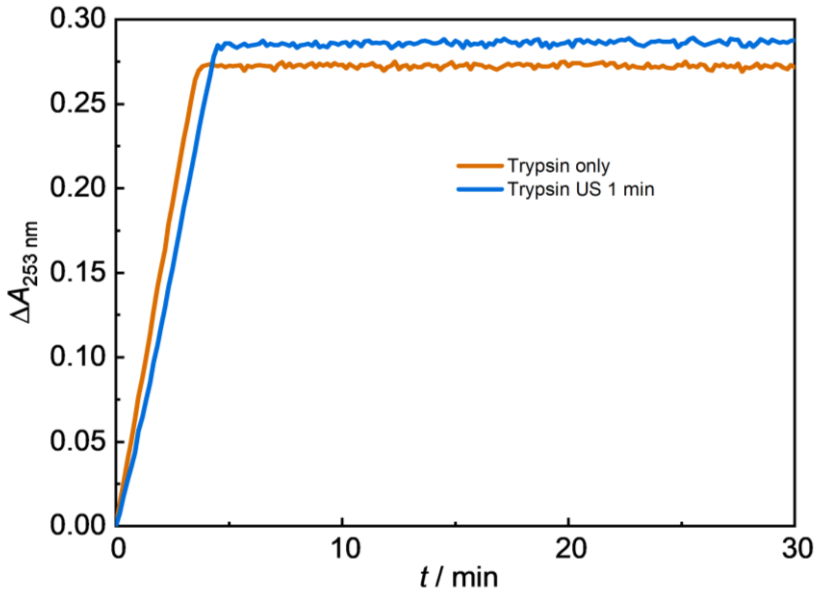


Figure S29. Trypsin remained at about 80% activity after 1 min sonication according to the enzymatic reactions using BAEE as substrate.

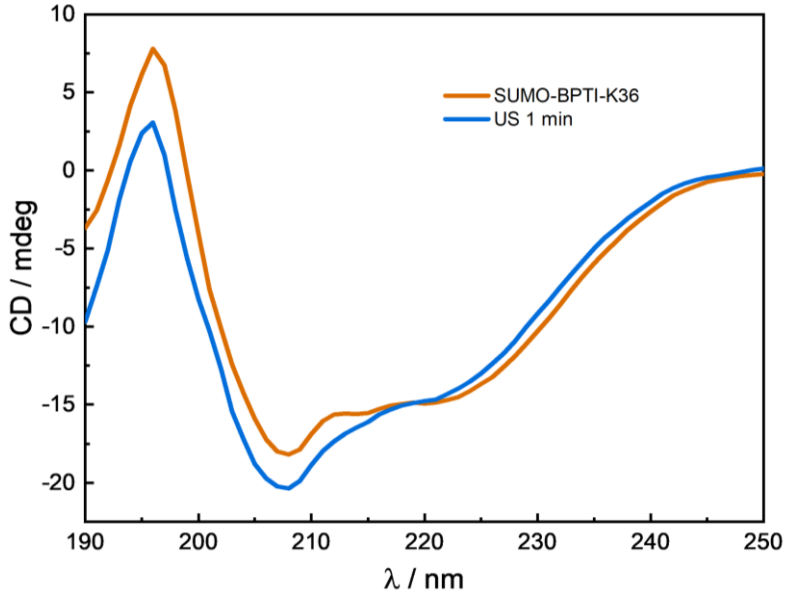


Figure S30. CD spectra of 50 μM SUMO-BPTI-K36 after 1 min sonication. Spectra were obtained in PBS buffer (pH = 7.4) at room temperature with a HT voltage lower than 500 V.

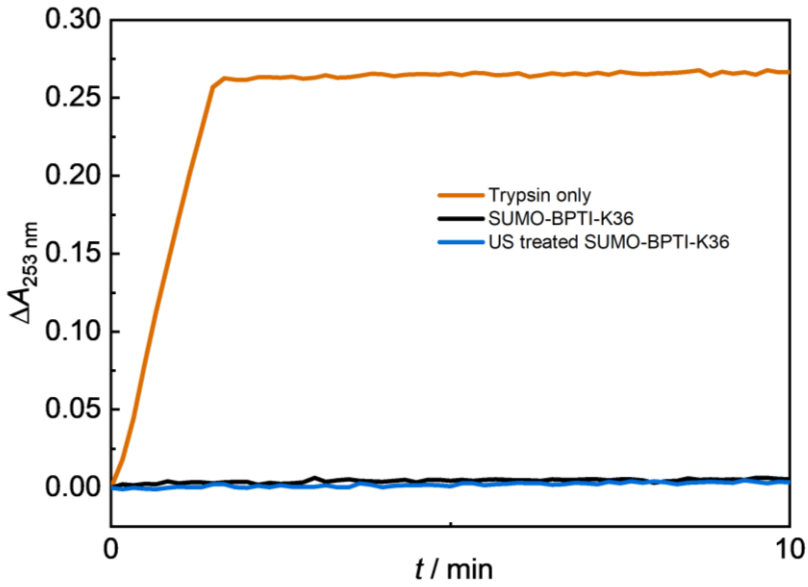


Figure S31. US treated SUMO-BPTI-K36 after 1 min still can inhibit trypsin (molar ratio between enzyme and inhibitor is 1:4) thoroughly according to the enzymatic reactions using BAEE as substrate.

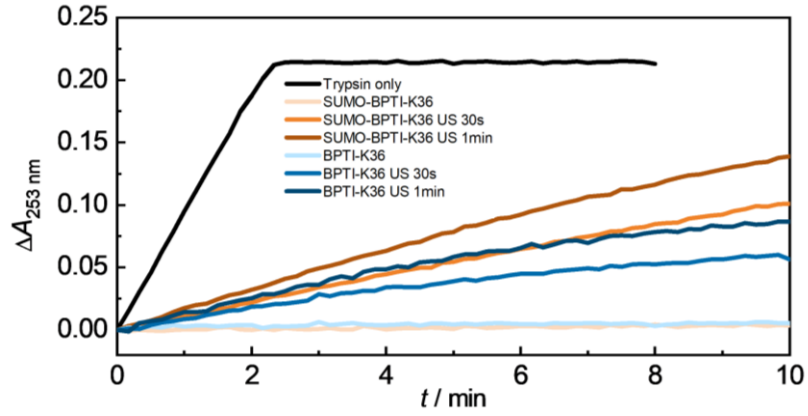


Figure S32. The K36 tail, and not the SUMO tag, plays an essential role in the ultrasound sensitivity of the trypsin-BPTI complexes. In the enzymatic reactions using BAEE as substrate, the BPTI-K36 blocks trypsin completely. The trypsin-BPTI-K36 complex is disassembled by ultrasound within seconds the same as SUMO-BPTI-K36. Conditions are as described in Figure 3.

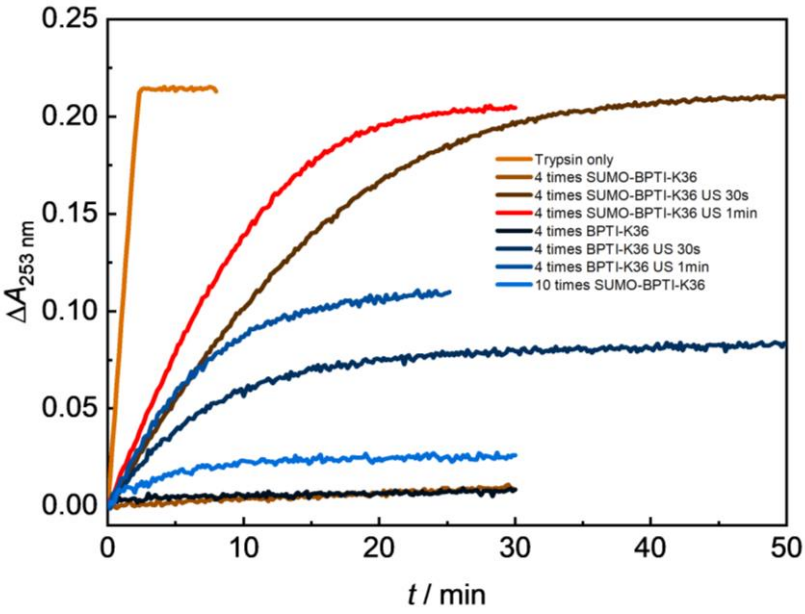


Figure S33. The enzymatic activity of trypsin, trypsin-SUMO-BPTI-K36, and trypsin-BPTI-K36 complexes for longer times of sonication. Trypsin-BPTI-K36 complexes showed lower conversion percentages because of BPTI-K36 has higher binding affinity. Also, higher trypsin inhibitors ratio of 1:10 was investigated to further elucidate the rebinding mechanism, showing that dynamic rebinding of an excess inhibitor reduces enzymatic activity.

References

[1] Kolbe, A.; del Mercato, L. L.; Abbasi, A. Z.; Rivera Gil, P.; Gorzini, S. J.; Huibers, W. H.; Poolman, B.; Parak, W. J.; Herrmann, A., De novo design of supercharged, unfolded protein polymers, and their assembly into supramolecular aggregates. *Macromol. Rapid Commun.* **2011**, 32 (2), 186-190.

[2] McDaniel, J. R.; Mackay, J. A.; Quiroz, F. G.; Chilkoti, A., Recursive directional ligation by plasmid reconstruction allows rapid and seamless cloning of oligomeric genes. *Biomacromolecules* **2010**, 11 (4), 944-952.

[3] Novagen **2004**. Competent cells user protocol. Novagen, Darmstadt, Germany.

[4] (a) Cabantous, S.; Terwilliger, T. C.; Waldo, G. S., Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nat. Biotechnol.* **2005**, 23 (1), 102-107; (b) Pedelacq, J. D.; Cabantous, S.; Tran, T.; Terwilliger, T. C.; Waldo, G. S., Engineering and characterization of a superfolder green fluorescent protein. *Nat. Biotechnol.* **2006**, 24 (1), 79-88.

[5] Sun, Z.; Lu, W.; Jiang, A.; Chen, J.; Tang, F.; Liu, J. N., Expression, purification and characterization of aprotinin and a human analogue of aprotinin. *Protein Expr. Purif.* **2009**, 65 (2), 238-243.

Author Contributions

A.H. and Y.Z. designed the study. Y.Z. performed all experiments. S.H. and M.L. provided reagents and advice. Y.Z., A.J.B., and R.G. analyzed and interpreted data. Y.Z., R.G., A.J.B., and A.H. designed figures and wrote the manuscript as well as the revision.