Supplementary Information

Redox-active chemical chaperones exhibiting promiscuous binding promote oxidative protein folding under condensed sub-millimolar conditions

Koki Suzuki,[a,†] Ryoya Nojiri,[a,†] Motonori Matsusaki,[b] Takuya Mabuchi,[c,d] Shingo Kanemura,[c] Kotone Ishii,[c] Hiroyuki Kumeta,[e] Masaki Okumura,*[c] Tomohide Saio,*[b] and Takahiro Muraoka*[a,f]

a Department of Applied Chemistry, Graduate School of Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan
E-mail: muraoka@go.tuat.ac.jp

b Division of Molecular Life Science, Institute of Advanced Medical Sciences, Tokushima University Tokushima 770-8503, Japan
E-mail: saio@tokushima-u.ac.jp

c Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan
E-mail: okmasaki@tohoku.ac.jp

d Institute of Fluid Science, Tohoku University, Sendai, Miyagi 980-8577, Japan

e Faculty of Advanced Life Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan

f Kanagawa Institute of Industrial Science and Technology (KISTEC), Kanagawa 243-0435, Japan

† These authors contributed equally to this work.

Table of Contents

1. Materials

Acetic anhydride, deuterated dimethyl sulfoxide (DMSO-d_6), deuterium oxide(D_2O) and dehydrated *N*,*N*-dimethylformamide (DMF) were purchased from Kanto Chemicals (Tokyo, Japan). Acetonitrile, Et_2O, 30% H_2O_2 aq., *N*-methyl-2-pyrrolidone (NMP), piperidine, sodium hydroxide (NaOH), and trifluoroacetic acid (TFA) were purchased from Kishida Chemical (Tokyo, Japan). Coomassie brilliant blue G-250, *N,N*-diisopropylethylamine (DIEA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1,4-dithiothreitol (DTT), L-glutathione oxidized (GSSG), L-glutathione reduced (GSH), guanidine hydrochloride (GdnHCl), and 6M hydrochloric acid and urea were purchased from Nacalai Tesque (Kyoto, Japan). Bovine pancreatic trypsin inhibitor (BPTI) was purchased from Pro-Spec-Tany TechnoGene (Rehovot, Israel). Cytidine 2':3'-cyclic monophosphate monosodium salt (cCMP), maleimidePEG-2000 (malPEG-2000) and ribonuclease A (RNase A) from bovine pancreas were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cystamine dihydrochloride, mono-2-*O*-(*p*-toluenesulfonyl)-*β*-cyclodextrin, mono-6-*O*-(*p*-toluenesulfonyl)-*β*-cyclodextrin, TNBS Test Kit and triisopropylsilane (TIS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Fmoc-Ala-OH•H_2O, Fmoc-Glu(OtBu)-OH•nH_2O, Fmoc-Gly-OH, Fmoc-Pro-OH•nH_2O, Fmoc-NH-SAL Resin, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, HBTU and HOBt•H_2O were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Deionized water (filtered through a 0.22 μm membrane filter, >18.2 MΩ cm) was purified in Purelab DV35 of ELGA (Buckinghamshire, UK) and a Milli-Q system of Merck Millipore (Burlington, MA, USA). βCD_NSH was synthesized by following the reported procedure.[21]

2. Instrumentation

Nuclear magnetic resonance (NMR) spectra were recorded on JNM-ECX 400 spectrometer (400 MHz for ^{1}H) or JNM-ECA 500 spectrometer (500 MHz for ^{1}H) of JEOL (Tokyo, Japan), where the chemical shifts (δ) were determined with respect to the resonances corresponding to the residual non-deuterated solvent for ^{1}H (DMSO-d_6: 2.54 ppm, D_2O: 4.67 ppm). NMR spectra for ^{15}N BPTI were recorded on Bruker spectrometer (500 MHz for ^{1}H). High-resolution matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF MS) spectra were recorded on autoflex speed of Bruker. UV-vis absorption spectra were recorded on V-750 UV-Vis spectrophotometer of JASCO (Tokyo, Japan). Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was conducted with Primaide HPLC system of HITACHI (Tokyo, Japan) using TSKgel Protein C4-300 column of Tosoh Bioscience (φ4.6 × 150 mm, Tokyo, Japan) for BPTI folding assay, and JASCO HPLC system using YMC Triart C18 column (φ4.6 × 250 mm, Kyoto, Japan) for redox potential measurements. Semi-preparative reversed-phase high-performance liquid chromatography performed on PU-4086- Binary pump, UV-4075 detector and CHF122SC fraction collector of JASCO (Tokyo, Japan) attached with TA12S05-2520WX Actus Triart column of YMC (φ20 × 250 mm, Tokyo, Japan).

3. Methods

1) Determination of thiol concentration for oxidative protein folding assay: A thiol compound dissolved in 10 mM HCl aq. was diluted in a buffer (50 mM Tris-HCl, 0.3 M NaCl, pH 7.5). The mixture was added to an aqueous solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and incubated for 10 min at 30 °C. The concentration of the thiol compound was determined by the absorbance at 412 nm measured at 30 ºC with V-750 UV-Vis spectrophotometer.[22]

2) Preparation of reduced and denatured RNase A: RNase A (32 mg mL^{-1}) was dissolved in a buffer (500 µL, 200 mM Tris-HCl, pH 8.7) containing 6.0 M GdnHCl and 100 mM DTT and the mixture was incubated for 2 h at 25 °C. The resulting mixture was dialyzed three times for 2 h each with 10 mM HCl aq. (1 L) to remove the denaturing and reducing reagents. After the dialysis, the sample was stored at –30 ºC until use.

3) RNase A refolding assay: Prior to the assay, a mixture of a disulfide compound and a thiol compound was dissolved in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5), and the resulting mixture was preincubated for 5 min at 30 °C. To the preincubated mixture, fully reduced and denatured RNase A (100 µM) was added and incubated at 30 °C in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5) containing 400 µM disulfide compound with 800 µM thiol compound.[16] At 30, 60, 120 and 180 min after starting the incubation, aliquots (10 µL each) were taken from the reaction solution and were immediately added to a buffer (490 µL, 50 mM Tris-HCl, 300 mM NaCl, pH 7.5) containing cCMP (final concentration of cCMP = 0.60 mM) followed by the measurement of the linear increase in absorbance at 284 nm at 30 °C with V-750 UV-Vis spectrophotometer. Values represent means ± SEM based on the three independent experiments.

4) Gel shift assay of RNase A folding: Oxidative folding of RNase A (100 µM) was carried out in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5) containing 400 µM disulfide compound with 800 µM thiol compound. At 0, 1, 5, 10, 30, 60, 120 and 180 min after starting the incubation, free thiols were inactivated by the addition of Laemmli's 4×SDS-loading buffer[23] containing 10 mM malPEG-2000. RNase A was separated depending on the number of disulfide bonds by non-reducing 14% SDS-PAGE using WIDE RANGE gel (Nacalai Tesque,

Kyoto, Japan). Proteins were visualized by coomassie brilliant blue staining. The gel image was imported with a ChemiDoc Touch Imaging System and the band intensities were analyzed by Image Lab software (Bio-Rad, Hercules, CA, USA).

5) BPTI folding assay: BPTI (10 mg) dissolved in 0.1 M Tris (pH 8.0, 1.0 mL) containing 30 mM DTT and 8 M urea was incubated for 3 h at 50 °C. Fully reduced and denatured BPTI was purified by RP-HPLC using an InertSustain C18 column (φ4.6 × 250 mm, GL Sciences, Tokyo Japan), and the collected fractions were lyophilized. The obtained powder was stored at –30 °C until use. For the assay, a mixture of a disulfide compound and a thiol compound was dissolved in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5), and the resulting mixture was preincubated for 10 min at 30 °C. To the preincubated mixture, fully reduced and denatured BPTI (600 μM) dissolved in 6 M urea containing 0.05% trifluoroacetic acid was added (final concentration of BPTI: 30 μM, urea: 300 mM, disulfide compound: 90 μM, thiol compound: 450 μM, Tris-HCl: 50 mM, NaCl: 300 mM, pH 7.5). After the incubation at 30 °C for predetermined periods, the reaction was quenched by adding an equal volume of 1 M HCl aq., which was then analyzed by RP-HPLC at a flow rate of 1.0 mL min^{-1} monitoring at 229 nm with a linear gradient of elutions (solvent A: 0.05% trifluoroacetic acid in water; solvent B: 0.05% trifluoroacetic acid in acetonitrile; percentages of solvent A: 95% at 0 min, 80% at 15 min, 30% at 115 min).

6) Determination of thiol pK_a values: Stock solutions of citric buffer (sodium citrate and HCl for pH 2.0–4.0), phosphate buffer (Na_2HPO_4 and KH_2PO_4 for pH 5.0–8.0), borate buffers ($Na_2B_4O_7$ and HCl for pH 8.5–9.0, $Na_2B_4O_7$ and NaOH for pH 10.0–13.0) were degassed with N_2 for 1 h immediately prior to use. A stock solution of a thiol compound in degassed water (5.0 mM) were then prepared. Immediately after the aqueous solution of the thiol compound (12.5 μL) and a buffer (487.5 μL) were combined in a 1-cm thick quartz cuvette, the UV absorption spectrum of the sample was measured. The pH value of the sample was measured by a HORIBA pH meter (9618S-10D), which had been calibrated prior to use with pH 4.01, 6.86 and 9.18 standard solutions (HORIBA 101-S). Absorbance values at 240 nm of the samples were plotted in the function of the pH values, and the pK_a value of the thiol compound was calculated with KaleidaGraph software (version 5.0.4) by a curve fitting analysis using eq. (1):

$$y = \frac{a + \frac{m_1}{10^{-x} \cdot b} + \frac{m_1 \cdot m_2}{10^{-2x} \cdot c}}{1 + \frac{m_1}{10^{-x}} + \frac{m_1 \cdot m_2}{10^{-2x}}};\ m_1 = K_{a1}\ ;m_2 = K_{a2} \qquad (1)$$

where a is the minimum value of absorbance in the measurement range, b is the value of absorbance at the inflection point of the graph, and c is the difference between the maximum and minimum values of absorbance in the measurement range. m_1 and m_2 are the expected acid dissociation constants K_{a1} and K_{a2}. In this case, the first curve fitting was performed using the initial values of m_1 = 0.0000001 and m_2 = 0.0000000001, which the software uses in its calculations, and the second curve fitting was performed using the obtained values of m_1 and m_2. Once again, the value of m_2 obtained was treated as the acid dissociation constant K_{a2} of the thiol compound, and the pK_a was calculated.

7) Redox potential *E*°' measurements: *E*°' values of the thiol compounds were determined by following the protocol described in a previous paper.[11i] A buffer (100 mM Tris-HCl, 1.0 mM EDTA, pH 7.0) was degassed with N_2 for longer than 1 h prior to use. DTT^{red} (60 μM, 4.0 mL) in the buffer was added to a disulfide compound (60 μM, 4.0 mL) in the buffer under N_2 at 25 ± 0.1 °C for 10 h. To quench the reaction, an aliquot of the reaction mixture (1.0 mL) was added to 1 M HCl aq. (200 μL), and the obtained solution was immediately analyzed by RP-HPLC (YMC Triart C18 column, φ4.6 × 250 mm). The RP-HPLC analysis was conducted with water/acetonitrile = 98/2 containing 0.1% TFA. The concentrations of the species at equilibrium were calculated from the observed peak areas and the corresponding calibration curves.

The equilibrium constant K_{eq} for the reaction (eq. 2), described as eq. 3, was determined by averaging seven times of individual experiments following the above procedure.

$$\text{Disulfide} + DTT^{red} \rightleftharpoons 2\text{Thiol} + DTT^{ox} \qquad (2)$$

$$K_{eq} = \frac{[\text{Thiol}]^2[DTT^{ox}]}{[\text{Disulfide}][DTT^{red}]} \qquad (3)$$

The redox potential *E*°′ was calculated by the Nernst's equation (eq. 4)

$$E^{\circ\prime} = E^{\circ\prime}_{DTT} + \frac{RT}{nF} \ln K_{eq} \qquad (4)$$

where, n is the number of transferred electrons (n = 2), F is Faraday's constant (96500 C mol^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the temperature (298 K), and $E^{0\prime}_{DTT}$ is the redox potential of DTT (–327 mV).

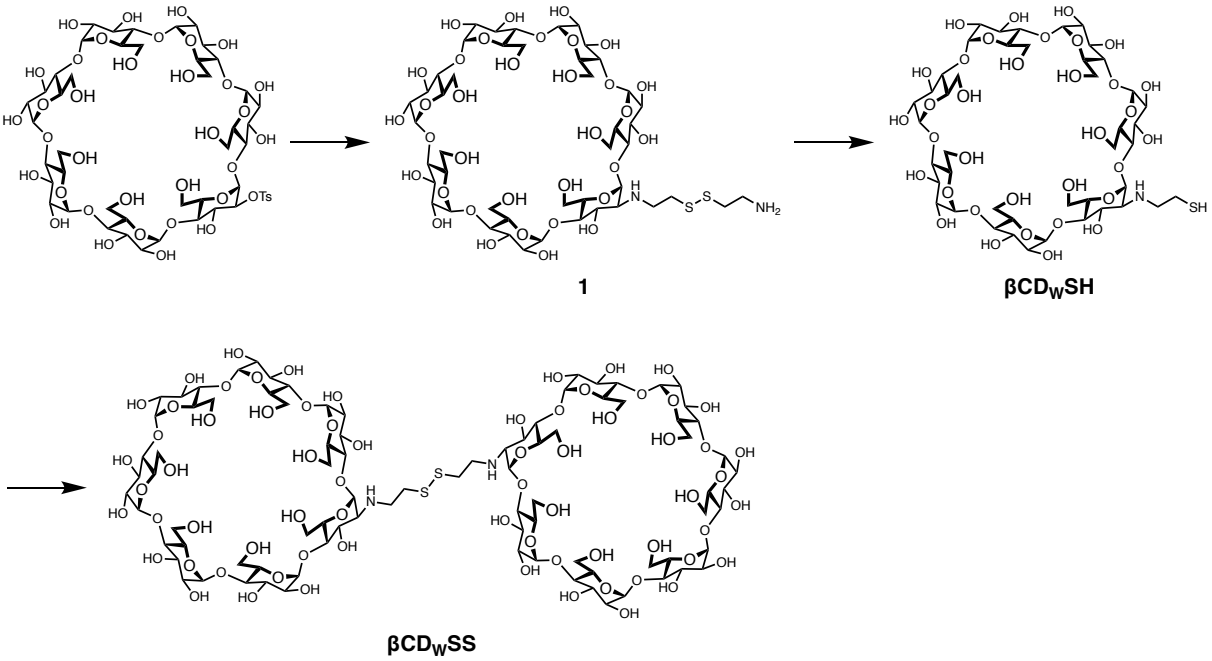
8) NMR interaction study for BPTI All-Ser: ^{15}N-labeled BPTI All-Ser were prepared at 0.1 mM concentration in 50 mM HEPES-NaOH pH 7.0, 10% $^{2}H_2O$, and subjected to NMR measurement on Bruker Avance III 500 MHz NMR spectrometer equipped with BBO probe with sample temperature of 10°C. ^{1}H–^{15}N selective optimized flip angle short transient (SOFAST)-heteronuclear multiple quantum correlation (HMQC) spectra[17] of ^{15}N-labeled BPTI All-Ser were acquired in the absence and presence of βCD, βCD$_W$SH, and βCD$_N$SH at 1, 3, 5 mM. The spectra were processed using NMRPipe program[24] and analyzed using Olivia program (https://github.com/yokochi47/Olivia). Amide resonance assignments for BPTI All-Ser were obtained with the reference of the previous study.[9]

9) Expression and purification of the recombinant BPTI All-Ser: cDNA encoding BPTI were subcloned into the NdeI and EcoRI sites of the pET17b vector (Novagen, Germany). BPTI mutant in which all the cysteine residues were replaced with serine residues (BPTI All-Ser) were constructed by using a PrimeSTAR Mutagenesis Basal Kit (Takara Bio, Japan). BPTI All-Ser was overexpressed in *Escherichia coli* strain BL21(DE3) by culturing at 37°C overnight. The cells were disrupted in the buffer containing 50 mM Tris-HCl (pH 8.1), 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride using a homogenizer (Sonics and Materials, USA). By the centrifugation of the homogenized lysate, recombinant BPTI all-Ser was obtained as an inclusion body. The inclusion body was dissolved in 100 mM Tris-HCl (pH 8.0) containing 8 M urea and 20 mM dithiothreitol at 50°C for 1 h. After centrifugation, the supernatant was loaded onto a COSMOSIL 5C18 column (Nacalai Tesque, Japan), and BPTI All-Ser was eluted with the 80% CH_3CN in 0.05% TFA. The eluted samples were finally purified by the RP-HPLC (GL Science, Japan) equipped with a COSMOSIL 5C18-AR-II column (Nacalai Tesque, Japan) using a linear gradient of CH_3CN in 0.05% TFA. Purified samples were lyophilized.[9]

10) MD simulations of βCD binding on unfolded BPTI: All-atom molecular dynamics (MD) simulations were performed with the CHARMM36m force field[25] using the LAMMPS software.[26] Three systems were constructed, each consisting of one unfolded BPTI molecule and five molecules of βCD, βCD$_W$SH, and βCD$_N$SH, respectively, in a periodic simulation box. The systems were solvated by adding water. The salt concentration was set to 300 mM NaCl,

and the corresponding numbers of Na^+ and Cl^- ions were added. Each system underwent a 100 ns NPT run at a temperature of 300 K and a pressure of 1 atm, and the trajectory data from the last 70 ns was used for analyses. The temperature was maintained with a Nosé–Hoover thermostat[27] and the pressure was controlled with a Parrinello–Rahman barostat.[28] The nonbonded interactions were calculated with a cutoff distance of 1.2 nm, and the particle−particle particle−mesh (PPPM) method[29] was used to calculate long-range electrostatic interactions. The equations of motion were integrated using the Verlet algorithm[30] with a time step of 2 fs, along with the SHAKE algorithm[31] to constrain the lengths of bonds to hydrogen. For analyses, the time-averaged fraction of contacts between βCD and each BPTI residue was calculated using *dr_sasa* software[32] with a probe diameter of 0.14 nm. The contact side of βCD with BPTI was defined as the one exhibiting a larger contact area between the narrow and wide sides of βCD, excluding the SH group, at each trajectory. PyMOL was used to generate the images.

4. Synthesis



Scheme S1. Synthetic scheme of βCDwSH.

1) Synthesis of 1: To a DMSO solution (2.0 mL) of mono-2-*O*-(*p*-toluenesulfonyl)-*β*-cyclodextrin (101 mg, 0.0780 mmol) was added dropwise an aqueous solution (0.50 mL) of cystamine dihydrochloride (175 mg, 0.778 mmol) and NaOH (65 mg, 1.63 mmol), and the mixture was stirred for 26 h at 80 °C. After cooling to room temperature, the reaction mixture was filtrated. The filtrate was added dropwise to an excess amount of acetone, and the precipitates were recovered by centrifugation. After repeating this process with EtOH (twice) and acetone (twice), the precipitates were dried under vacuum at 60 °C to obtain **1** in quantitative yield (103 mg).

^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): δ = 5.89–5.35 (m, 13H), 5.02–4.50 (m, 14H), 3.93–3.53 (m, 28H), 2.99–2.76 (m, 8H) ppm; MALDI-TOF MS (gentisic acid, positive mode): *m*/*z* = 1291.386 (calculated *m*/*z* on the basis of the monoisotopic mass of $C_{46}H_{80}N_2NaO_{34}S_2$ $[M + Na]^+$ = 1291.393).

2) Synthesis of βCDwSH: To a dry and degassed DMF solution (0.75 mL) of **1** (114 mg, 0.0897 mmol) was added a degassed aqueous solution (0.25 mL) of dithiothreitol (28 mg, 0.18 mmol)

under N_2. After stirring at 25 °C for 20 h, the reaction mixture was filtrated. The filtrate was added dropwise to an excess amount of acetone, and the precipitates were recovered by centrifugation. After repeating this process three times, the precipitates were dried under vacuum to obtain βCD_WSH in 15% yield (17 mg).

^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ = 5.85–5.53 (m, 13H), 4.95–4.40 (m, 14H), 3.70–3.49 (m, 28H), 2.88–2.69 (m, 4H) ppm; MALDI-TOF MS (gentisic acid, positive mode): *m*/*z* = 1216.388 (calculated *m*/*z* on the basis of the monoisotopic mass of $C_{44}H_{75}NNaO_{34}S$ $[M + Na]^+$ = 1216.379).

3) Synthesis of βCD_WSS: To an aqueous solution (1.0 mL) of βCD_WSH (100 mg, 0.0837 mmol) was added 30% H_2O_2 aq. (20 μL). After stirring at 25 °C for 24 h, the reaction mixture was chromatographed on Biotage Isolera One equipped with a Sfär Bio Silicagel Column (eluent: water/CH_3CN = 95/5 to 40/60 containing 0.1% TFA) to isolate βCD_WSS in 2% yield (4 mg).

^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ = 6.09–5.67 (m, 26H), 5.00–4.57 (m, 28H), 3.79–3.58 (m, 56H), 2.93–2.77 (m, 8H) ppm; MALDI-TOF MS (gentisic acid, positive mode): *m*/*z* = 2408.175 (calculated *m*/*z* on the basis of the monoisotopic mass of $C_{88}H_{148}N_2NaO_{68}S_2$ $[M + Na]^+$ = 2407.752).

4) Stock Solutions for Peptide Synthesis: A set of stock solutions of A) condensation-reagents: HBTU (3.05 g, 8.04 mmol) and HOBt•H_2O (1.25 g, 8.16 mmol) in DMF (16 mL), B) a mixture of DIEA (2.75 mL) and NMP (14.25 mL), and C) cleavage-reagents: TIS (62.5 μL), TFA (2.375 mL), and water (62.5 μL) were prepared just before the synthesis.

5) Synthesis of BPTI7-13 (Ac-EPPYTGP-NH_2), BPTI7-13(G12A) (Ac-EPPYTAP-NH_2), and BPTI7-13(G12V) (Ac-EPPYTVP-NH_2): Fmoc- NH-SAL Resin (0.10 mmol) was soaked in DMF (2 mL) overnight at 25 °C in a polypropylene tube, then DMF was removed. Piperidine in DMF (20%, 2 mL) was added to the soaked resin. After vortexing the mixture for 1 min, the reaction solution was removed, and this process was repeated one more time, followed by

washing the resin with DMF (2 mL, 5 times), CH_2Cl_2 (2 mL, 3 times), and DMF (2 mL, 3 times). Fmoc-protected amino acid (0.30 mmol) was dissolved in the stock solutions of A (700 μL) and B (700 μL). The obtained mixture was added to the resin, and the reaction mixture was shaken for 15 min at 25 °C followed by the removal of the reaction solution and washing the resin with DMF (2 mL, 5 times), CH_2Cl_2 (2 mL, 3 times) and DMF (2 mL, 3 times). The Fmoc-deprotection and subsequent coupling reactions were repeated by following the designed amino acid sequence. After the final Fmoc-deprotection reaction and washing of the resin, to the reaction was added tube acetic anhydride in CH_2Cl_2 (25%, 2 mL). The mixture was shaken for 5 min at 25 °C, and the reaction solution was removed, followed by washing of the resin with DMF (2 mL, 5 times), CH_2Cl_2 (2 mL, 3 times), and DMF (2 mL, 3 times). Then, the stock solution of C (2.5 mL) was added to the reaction tube, and the mixture was incubated for 90 min at 25 °C with gentle shaking every 30 min. The reaction solution was collected into a polypropylene centrifuge tube by filtration. The reaction tube was rinsed with TFA (500 μL, 3 times), and the solution was collected into the centrifuge tube by filtration. To the collected solution in the centrifuge tube was added Et_2O (40 mL), and the mixture was vortexed for 1 min, followed by centrifugation at 4 °C (3500 ×*g*, 5 min). Then, the supernatant liquid was removed by decantation. After repeating this process 3 times, the residue was dried under a vacuum for 2 h at 25 °C, and the residual peptide was dispersed in water, followed by lyophilization. the reaction mixture was chromatographed on preparative reversed-phase HPLC (eluent: water/CH_3CN = 100/0 to 40/60 containing 0.1% TFA) The collected samples were evaporated and lyophilized.

BPTI7-13: MALDI-TOF MS (gentisic acid, positive mode): *m*/*z* = 823.355 (calculated *m*/*z* based on the monoisotopic mass of $C_{37}H_{52}N_8NaO_{12}[M + Na]^+$ = 823.360).

BPTI7-13(G12A): MALDI-TOF MS (gentisic acid, positive mode): *m*/*z* = 837.389 (calculated *m*/*z* based on the monoisotopic mass of $C_{38}H_{54}N_8NaO_{12}[M + Na]^+$ = 837.376).

BPTI7-13(G12V): MALDI-TOF MS (gentisic acid, positive mode): *m*/*z* = 865.399 (calculated *m*/*z* based on the monoisotopic mass of $C_{40}H_{58}N_8NaO_{12}[M + Na]^+$ = 865.407).

5. Supplementary Figures

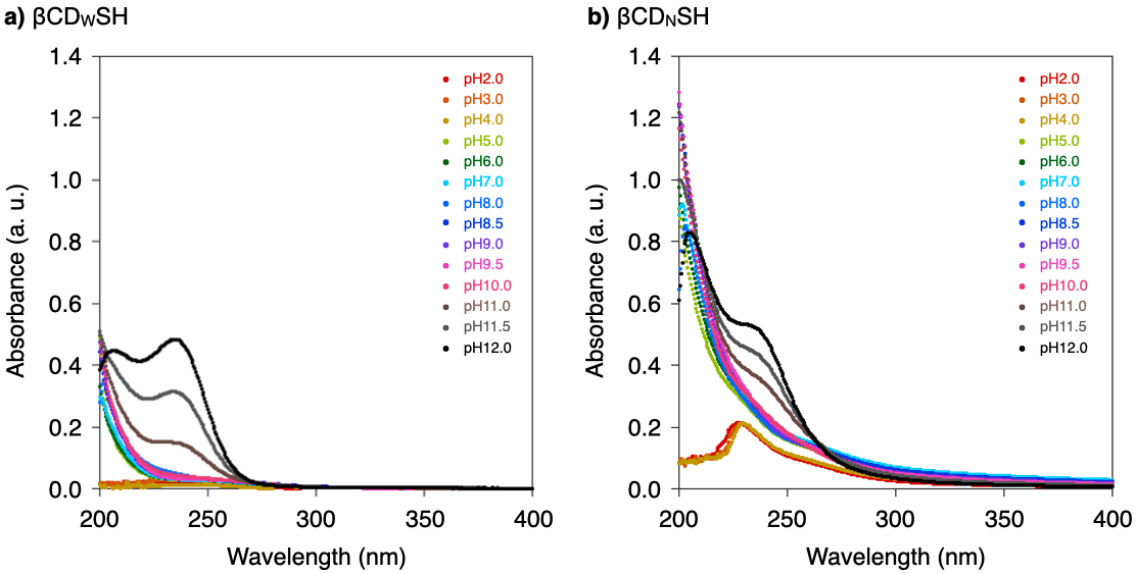


Figure S1. UV-vis absorption spectra of a) βCD_WSH and b) βCD_NSH at variable pH between 2.0 and 12.0. [Thiol compounds] = 125 µM, 25 °C.

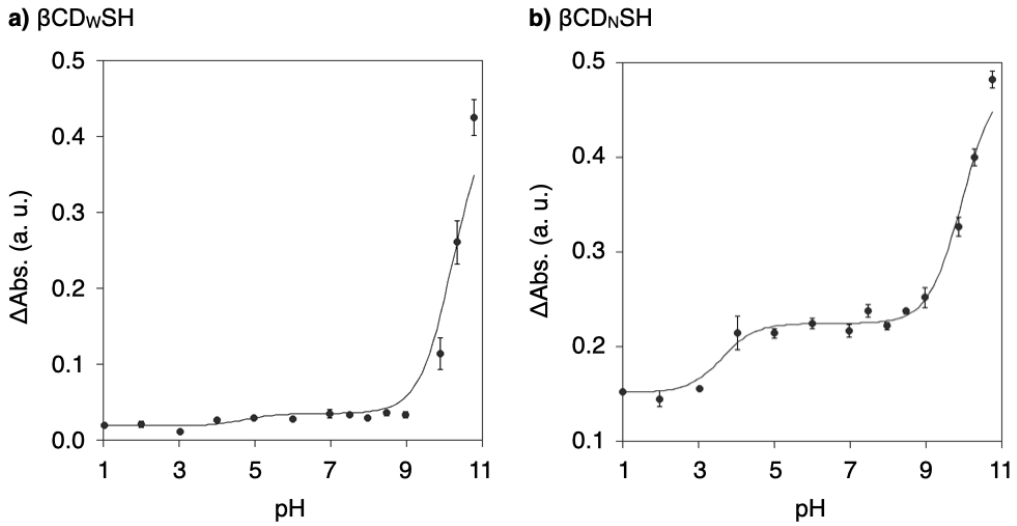


Figure S2. Absorbance changes at 240 nm and curve fitting analyses of a) βCD_WSH and b) βCD_NSH at variable pH between 2.0 and 12.0. [Thiol compounds] = 125 μM, 25 °C.

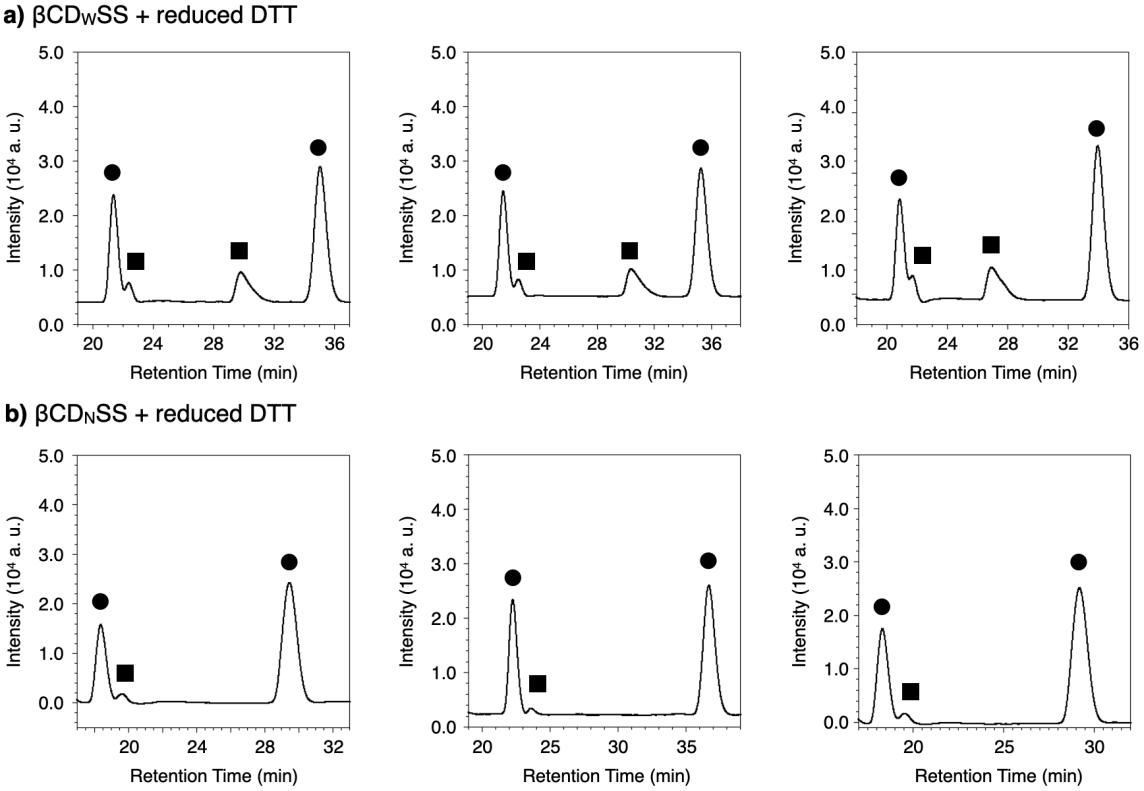


Figure S3. RP-HPLC trace of equilibrated mixtures of a) βCD_WSS and b) βCD_NSS with DTT^{red}. Reaction conditions were $[\beta CD_NSS]^0 = [DTT^{red}]^0 = 30$ μM at 25 °C and pH 7.0 for 10 h. Fractions marked with black circles correspond reduced and oxidized forms of DTT. Fractions marked with black squares correspond reduced and oxidized forms of βCD_WSS or βCD_NSS.

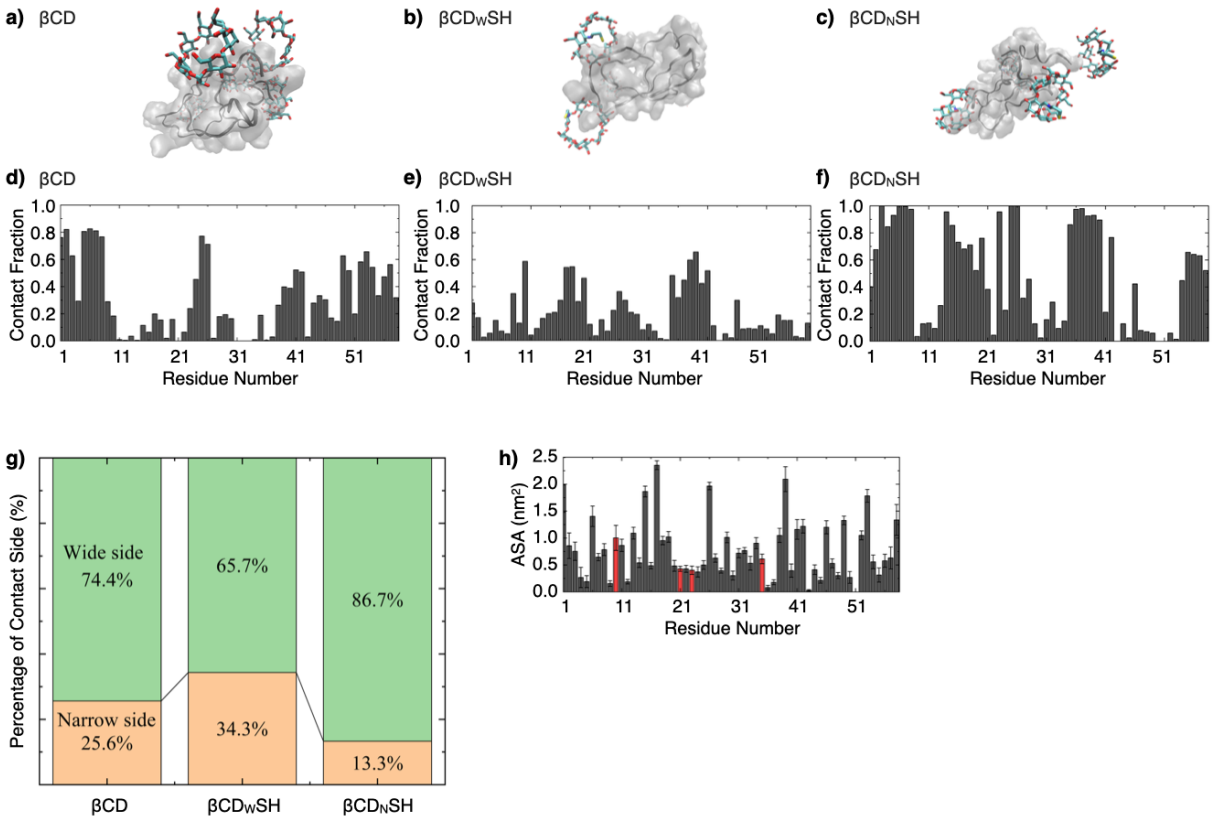


Figure S4. Molecular dynamics simulations over 100 ns of one reduced BPTI molecule in the presence of five molecules of a,d) βCD, b,e) βCD_WSH, and c,f) βCD_NSH in 300 mM NaCl aq. a–c) Representative snapshots. d–f) Time-averaged fraction of contacts between the additives (βCD, βCD_WSH, or βCD_NSH, respectively) and each BPTI residue. g) Time-averaged percentage of contact sides of βCD, βCD_WSH, and βCD_NSH over BPTI. h) Accessible surface area (ASA) for each residue of BPTI.

Figure S4 shows the results of additional simulations using the identical starting configuration of BPTI. The trends showing that βCD_WSH exhibits promiscuous binding while βCD and βCD_NSH exhibit preferential binding were found to be consistent with the original simulations shown in the main text. The ASA values for each residue were evaluated, and the order of ASA values was found to be Tyr10 > Tyr35 > Tyr23 > Tyr21 regardless of cyclodextrin types, which is consistent with the NMR analyses as well as the hydrophobic score (Figure S10c). The ASA values were not changed significantly during each simulation. The specific contact directly to the Tyr10 and Tyr35 was not necessarily observed in the simulations. This may be attributed to the complex interactions involving surrounding residues in the NMR results.

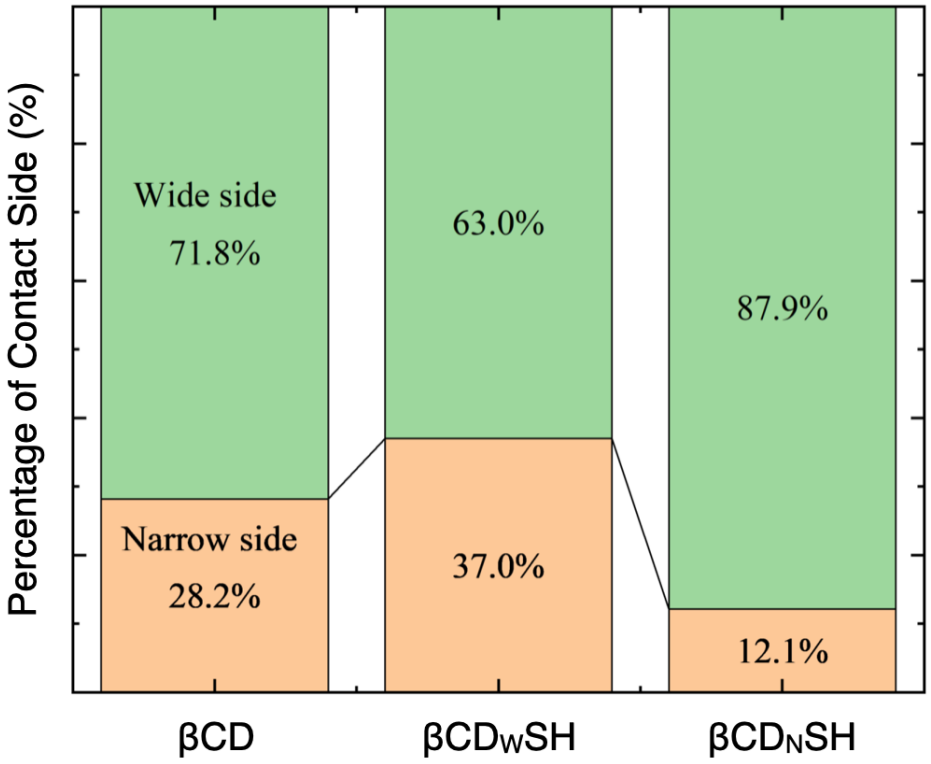
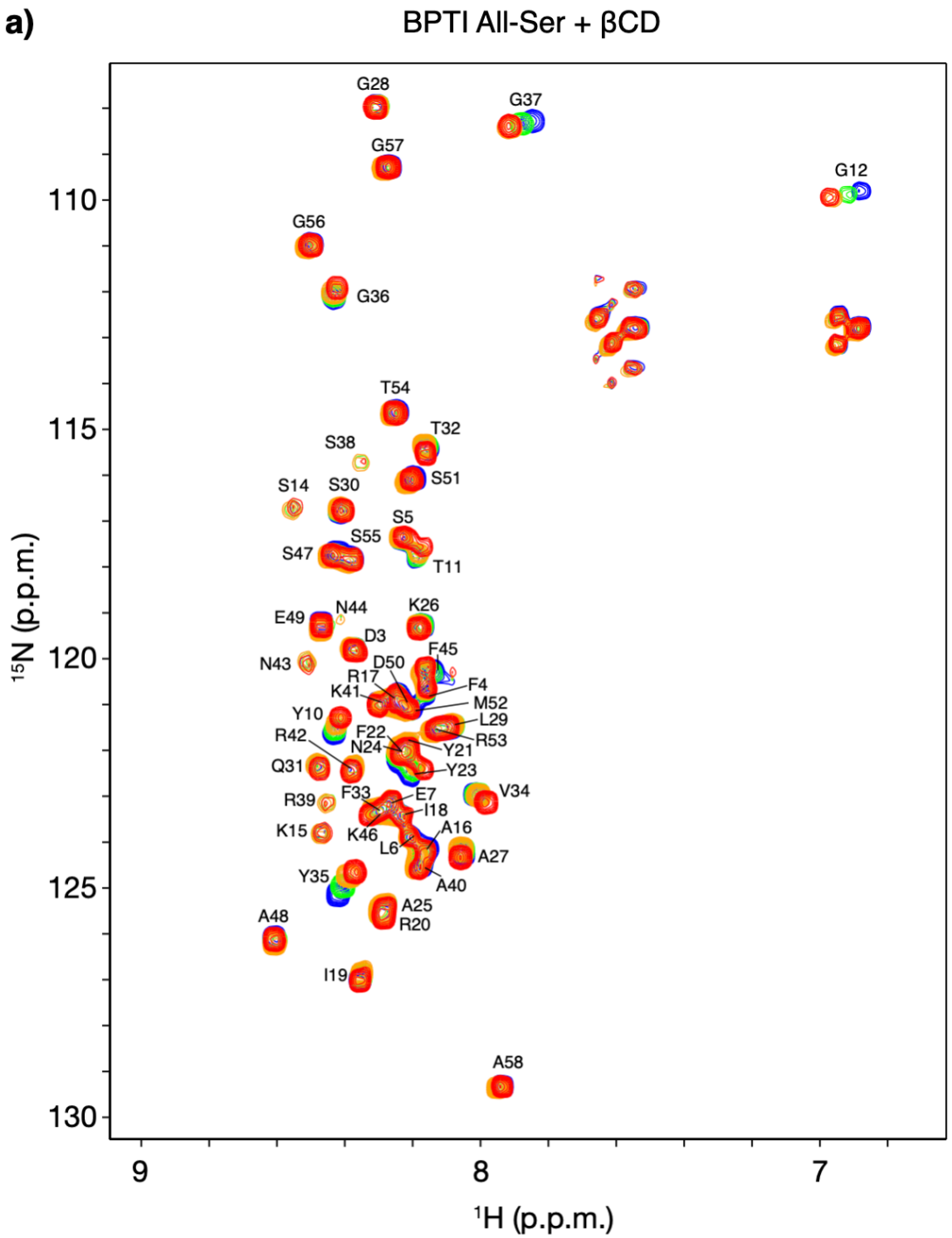
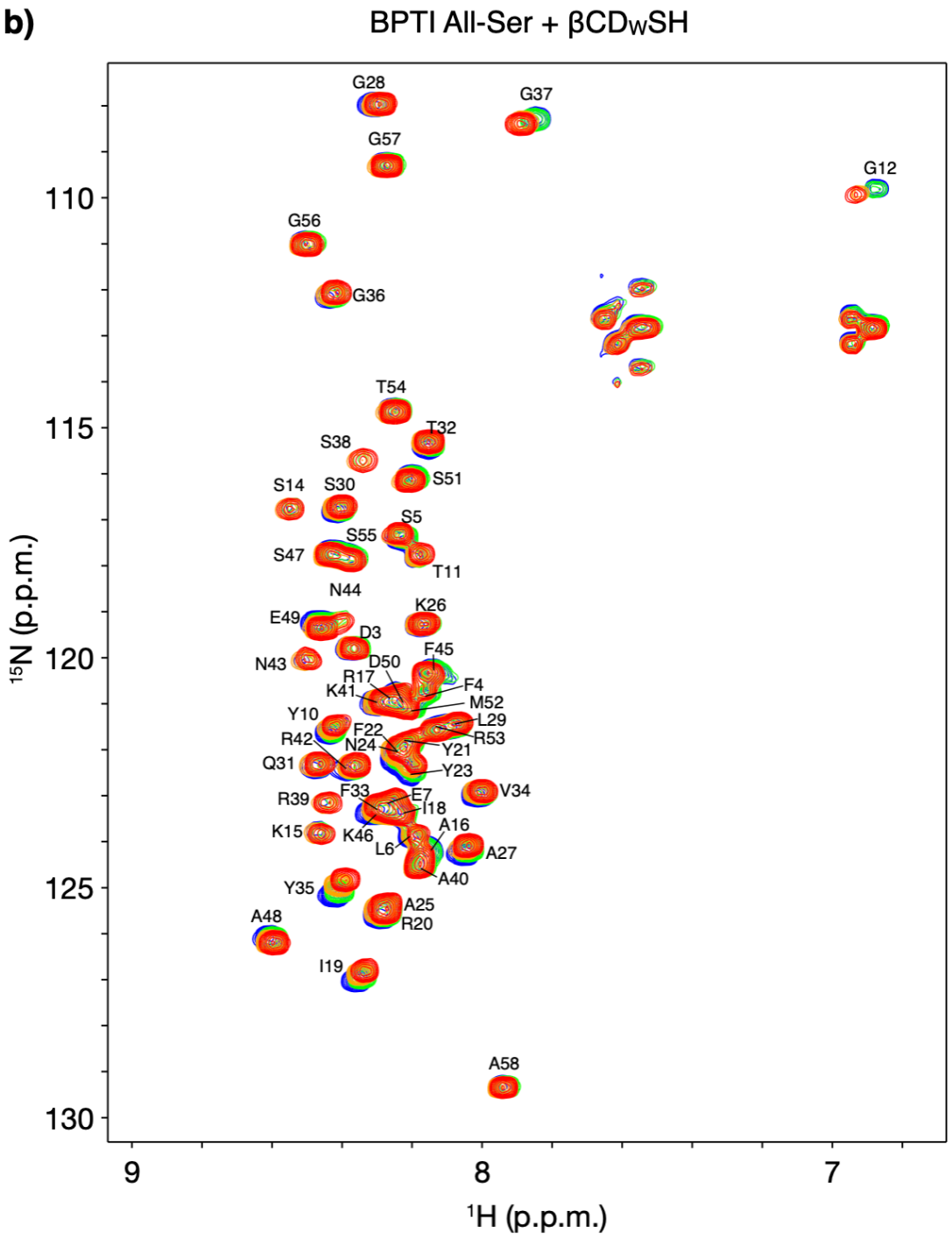


Figure S5. Time-averaged percentage of contact sides of βCD, βCD$_W$SH, and βCD$_N$SH over BPTI.


a)
BPTI All-Ser + βCD
G28
G37
G57
G12
G56
G36
T54
T32
S38
S51
S14
S30
S5
S55
S47
T11
E49
N44
K26
D3
N43
D50
F45
R17
F4
K41
M52
Y10
L29
R42
F22
R53
N24
Y21
Q31
Y23
R39
F33
E7
V34
I18
K15
K46
A16
L6
A27
Y35
A40
A25
A48
R20
I19
A58
110
115
120
125
130
^{15}N (p.p.m.)
9
8
7
^{1}H (p.p.m.)


b)
BPTI All-Ser + βCDwSH
G28
G37
G57
G12
G56
G36
T54
T32
S38
S51
S14
S30
S5
S55
S47
T11
N44
K26
E49
D3
N43
D50
F45
R17
F4
K41
M52
Y10
L29
F22
R42
N24
R53
Y21
Q31
Y23
F33
V34
R39
E7
I18
A16
K15
K46
L6
A27
A40
Y35
A25
A48
R20
I19
A58
110
115
120
125
130
9
8
7
15N (p.p.m.)
1H (p.p.m.)

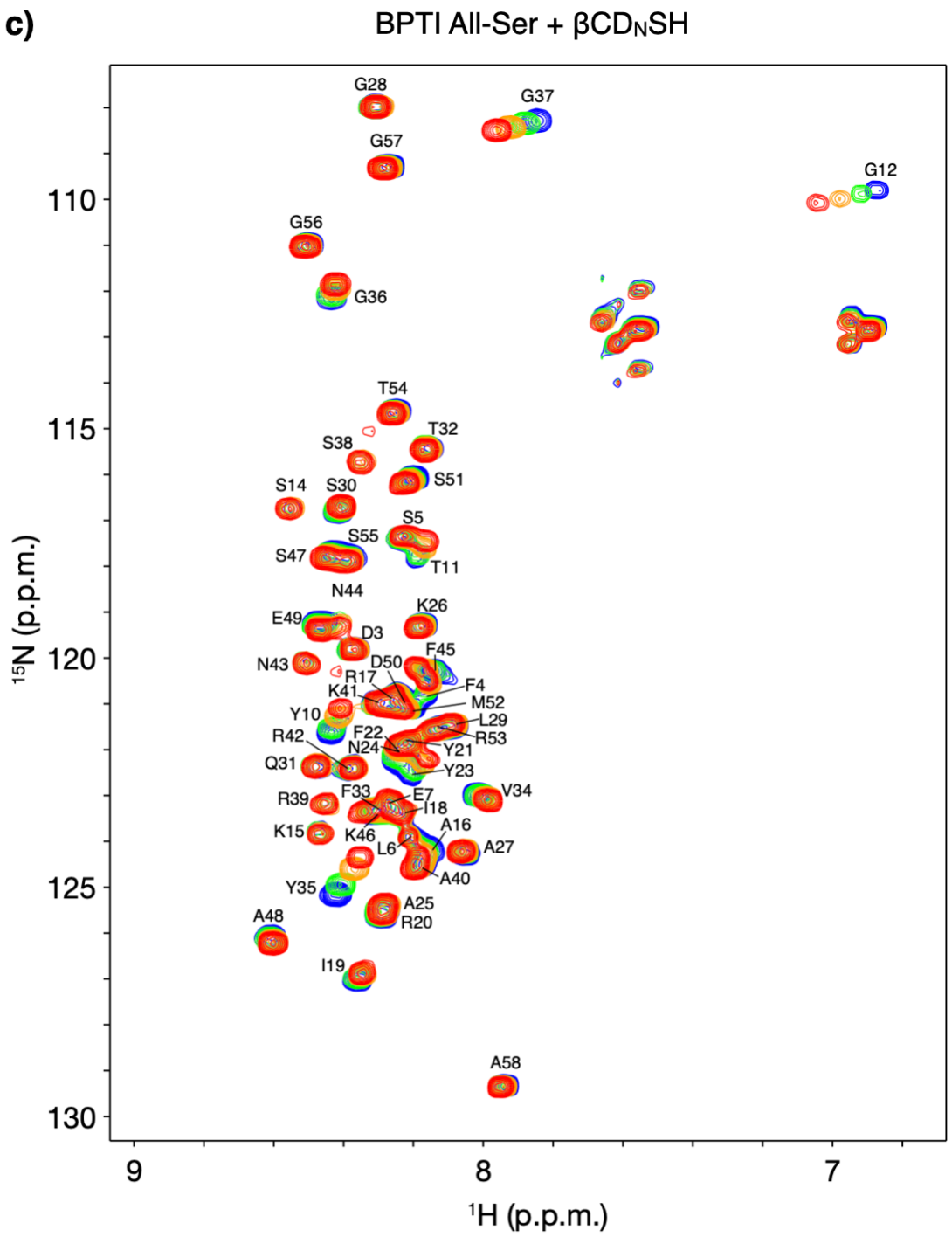


Figure S6. ^{1}H–^{15}N correlation SOFAST-HMQC spectra of ^{15}N BPTI All-Ser upon titration of a) βCD, b) $βCD_WSH$, and c) $βCD_NSH$. [^{15}N BPTI All-Ser] = 100 μM, 50 mM HEPES (pH7.0), 10 v/v% D_2O, 10 °C, 500 MHz. Concentration of additives: 0 mM (blue), 1 mM (green), 3 mM (orange), and 5 mM (red).

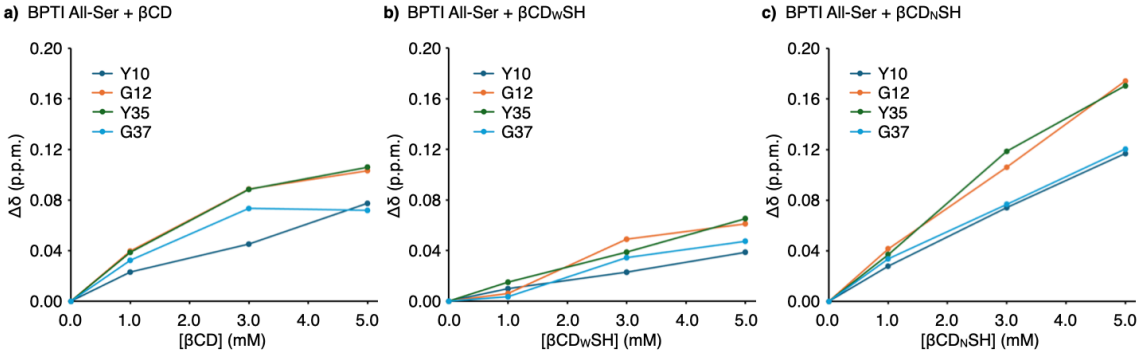


Figure S7. Averaged chemical shift changes Δδ of ^{1}H and ^{15}N signals corresponding to Y10, G12, Y35, and G37 upon addition of a) βCD, b) $\beta CD_{W}SH$, and c) $\beta CD_{N}SH$ in the ^{1}H-^{15}N correlation SOFAST HMQC spectra of ^{15}N BPTI All-Ser.

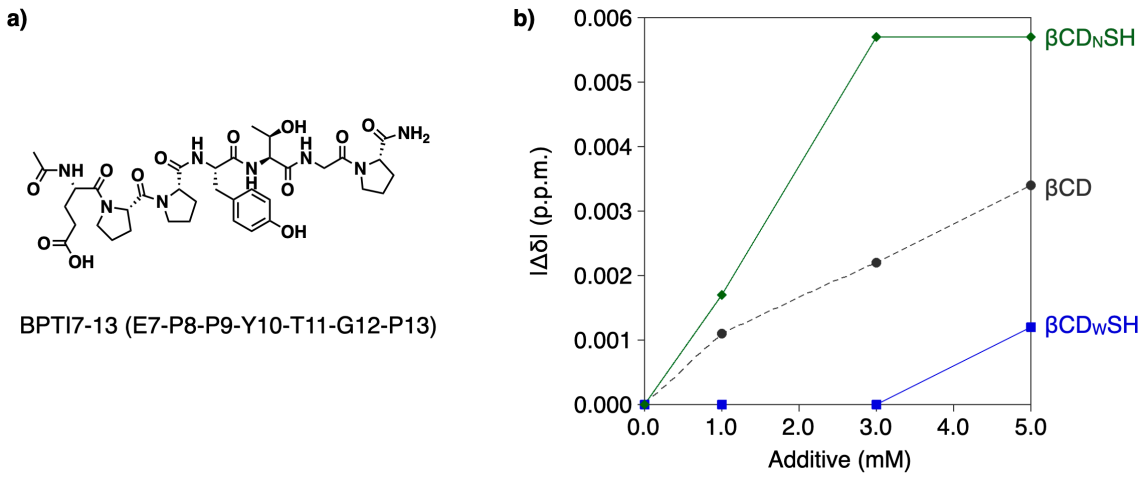


Figure S8. a) Chemical structure of peptide BPTI7-13 consisting of the same amino acid sequence at 7–13 residues of BPTI. b) Plots of Δδ changes at a signal corresponding to Tyr10 (the residual number corresponds to that in full length BPTI) of peptide BPTI7-13 upon titration with 0, 1, 3, and 5 mM of βCD, βCD_NSH, and βCD_WSH in D_2O. [BPTI7-13] = 100 μM, 25 °C, 500 MHz.

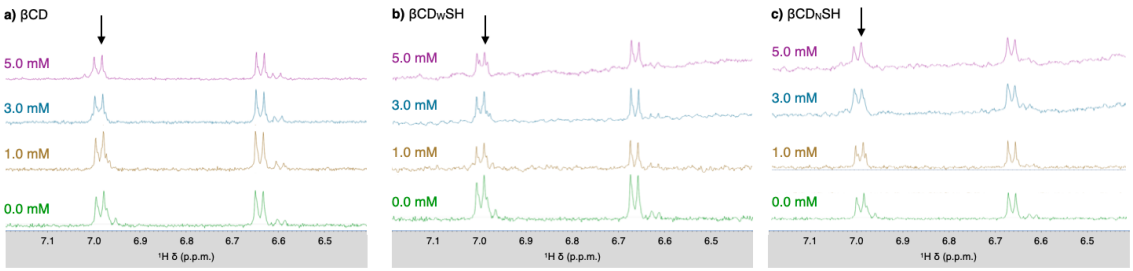


Figure S9. ^{1}H NMR spectra of BPTI7-13 upon titration with 0, 1, 3, and 5 mM of a) βCD, b) βCD_WSH, and c) βCD_NSH in D_2O. [BPTI7-13] = 100 μM, 25 °C, 500 MHz. The signals indicated by arrows were plotted in Figure S8.

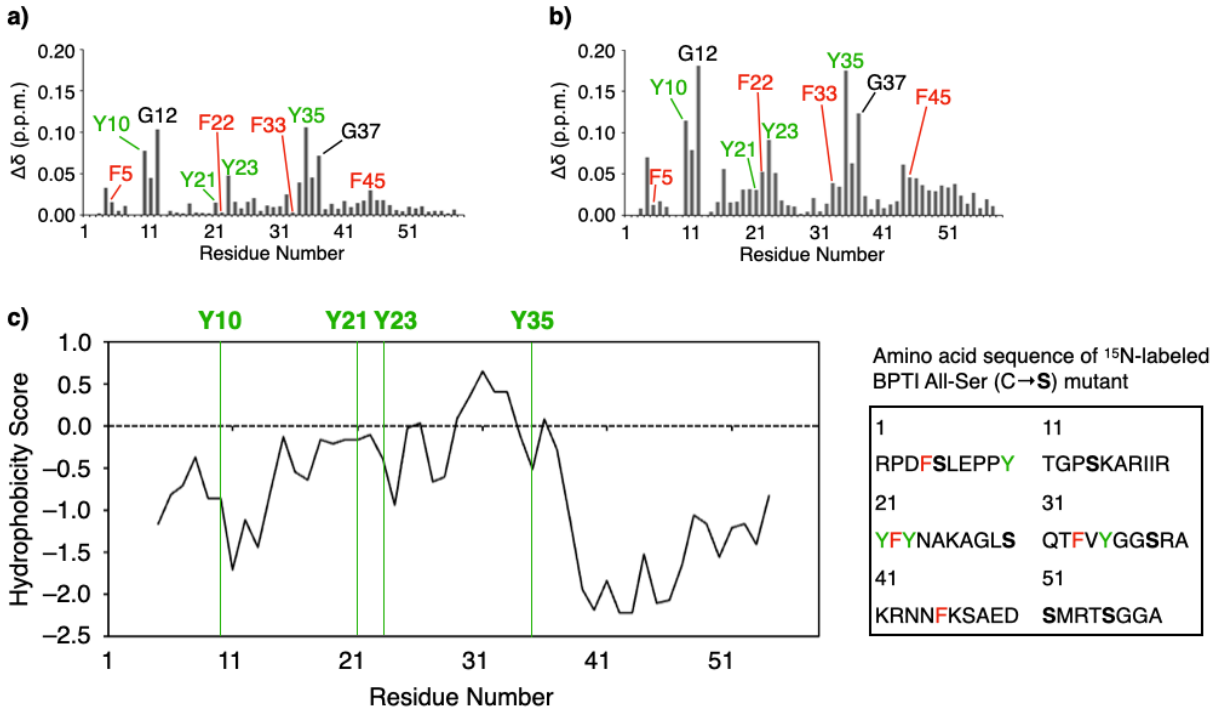


Figure S10. Chemical shift changes for ^{15}N BPTI All-Ser upon the addition of 5 mM a) βCD and b) βCD$_N$SH with marking the signals corresponding to Phe (red) and Tyr (green). [^{15}N BPTI All-Ser] = 100 μM, 50 mM HEPES (pH 7.0), 10 v/v% D_2O, 10 °C, 500 MHz. c) Plot of hydrophobicity score at the amino acid residues of ^{15}N-labeled BPTI All-Ser mutant. The positions of Tyr residues are highlighted. The plot was calculated at ProtScale (https://web.expasy.org/protscale/). Hydropath. / Kyte & Doolittle, Window size 9.

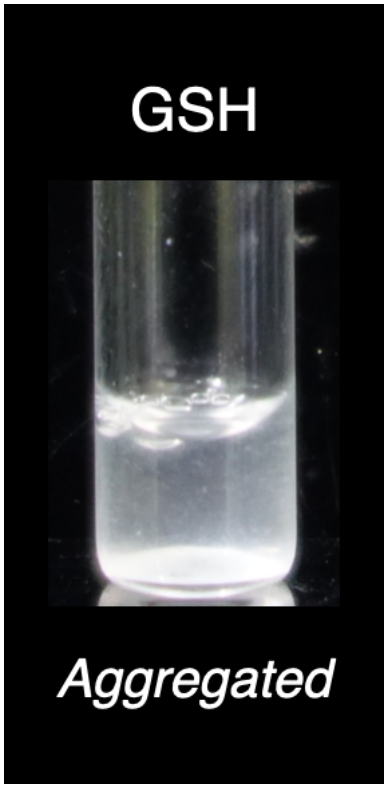


Figure S11. Photograph of unfolded and reduced BPTI (150 μM) in the presence of GSH and GSSG ([additives] = 1000 μM).

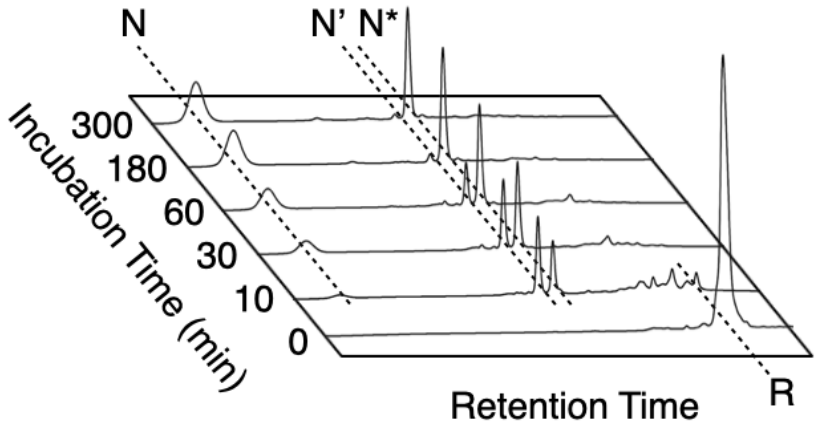


Figure S12. Time-course reverse-phase HPLC analysis of oxidative folding of BPTI (150 μM) in the presence of b) GSH (1.0 mM) and GSSG (450 μM) between 16- and 45-min retention time. N and R depict native and reduced forms of BPTI, respectively. N' and N* are folding intermediates. Eluent buffers: water (containing 0.05% TFA) and CH_3CN (containing 0.05% TFA) with a linear gradient; flow rate: 1.0 mL min^{-1}; detection wavelength: 229 nm; temperature: 50 °C.

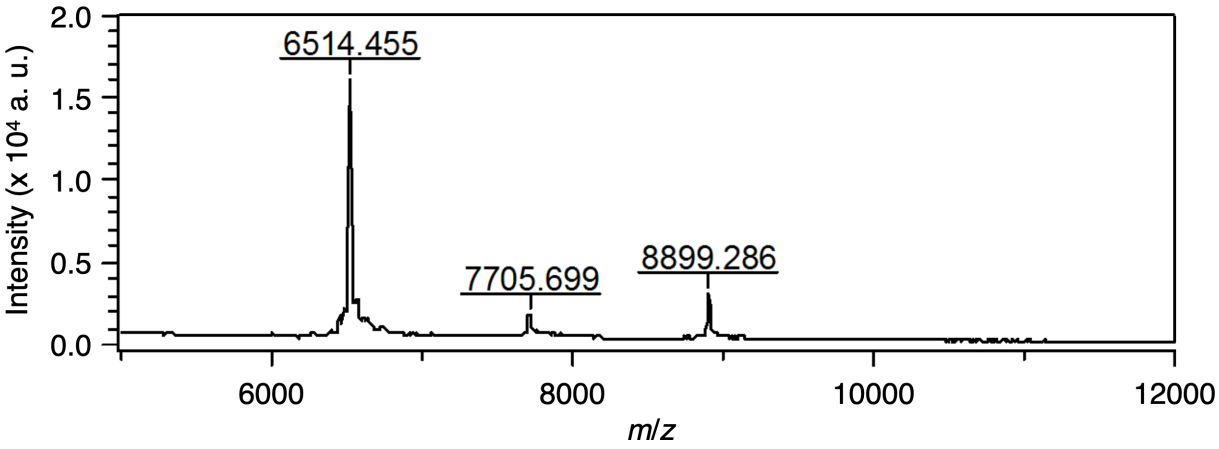


Figure S13. MALDI-TOF MS analyses of HPLC fractions between the N- and N'-fractions of BPTI (150 μM) obtained in the oxidative folding in the presence of βCD$_W$SH (1.0 mM) and βCD$_W$SS (450 μM) . The measurement was conducted in a linear positive mode using sinapic acid as the matrix. The observed signals at *m*/*z* = 6514, 7705, and 8899 can be assigned to BPTI, BPTI and βCD$_W$SH 1:1 complex, and BPTI and βCD$_W$SH 1:2 complex, respectively.

6. Supplementary References

21 Z. Chen, H. Cao and T. Tan, Preparation of functionalized star polymer nanoparticles by RAFT polymerization and their application in positionally assembled enzymes for cascade reactions, *New J. Chem.*, 2019, **43**, 8517-8526.

22 G. L. Ellman, Tissue sulfhydryl groups, *Arch. Biochem. Biophys.*, 1959, **82**, 70-77.

23 U. K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 1970, **227**, 680-685.

24 F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer and A. Bax, NMRPipe/ A multidimensional spectral processing system based on UNIX pipes, *J. Biomol. NMR*, 1995, **6**, 277-293.

25 J. Huang, S. Rauscher, G. Nawrocki, T. Ran, M. Feig, B. L. de Groot, H. Grubmüller and A. D. MacKerell, CHARMM36m: an improved force field for folded and intrinsically disordered proteins, *Nat. Methods*, 2017, **14**, 71-73.

26 S. Plimpton, Fast parallel algorithms for short-range molecular dynamics, *J. Comp. Phys.*, 1995, **117**, 1-19.

27 W. G. Hoover, Constant-pressure equations of motion, *Phys. Rev. A*, 1986, **34**, 2499.

28 G. J. Martyna, D. J. Tobias and M. L. Klein, Constant pressure molecular dynamics algorithms, *J. Chem. Phys.*, 1994, **101**, 4177-4189.

29 R. W. Hockney and J. W. Eastwood, *Computer Simulation Using Particles*, Hilger, Bristol, 1989.

30 L. Verlet, Computer "experiments" on classical fluids. I. Thermodynamical properties of Lennard-Jones molecules, *Phys. Rev.*, 1967, **159**, 98.

31 J.-P. Ryckaert, G. Ciccotti and H. J. C. Berendsen, Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes, *J. Comp. Phys.*, 1977, **23**, 327-341.

32 J. Ribeiro, C. Ríos-Vera, F. Melo and A. Schüller, Calculation of accurate interatomic contact surface areas for the quantitative analysis of non-bonded molecular interactions, *Bioinformatics*, 2019, **35**, 3499-3501.