Electronic Supplementary Material (ESI) for Molecular BioSystems.

Screening for Concanavalin A Binders from a Mannose-Modified α-Helix Peptide Phage Library

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Experimental section

General

All chemicals and solvents were of reagent or HPLC grade and were used without further purification. Concanavalin A and methyl α-D-mannopyranoside (Me-Man) were purchased from Sigma-Aldrich. Dynabeads M-280 Streptavidin was purchased from Life Technologies. RP-HPLC was performed on the Hitachi L7000 system using a Cosmosil 5C18-ARII (ϕ10×250 mm) column for purification with a linear gradient of acetonitrile/0.1% trifluoroacetic acid (TFA) at a flow rate of 3.0 mL/min. Electrospray ionization mass spectrometry (ESI-MS) was measured on a Shimadzu LCMS-2010 EV.

Construction of phage peptide library

The recombinant library plasmid (**fdg3p0ss21-library**) was constructed with a right semi-randomize DNA encoding library gene (**library insert**) inserted into the multiple cloning sites (MSC) of the disulfide-free pIII domains phage (**fd0D12**) vector.[S1,2] To minimize the transformation of cells with undigested and/or recircularized fd0D12 plasmid, a *Sfi1*-digested **fdg3p0ss21-stop** vector was utilized for the ligation with a *Sfi1*-digested library insert. The phage vectors fd0D12 and fdg3p0ss21-stop are the recipient vectors of fdg3p0ss21 and were prepared as reported.

Two consecutive polymerase chain reaction (PCR) steps were involved for the preparation of the DNA coding insert. [S1,2] In a first PCR reaction, the primers prepcrba and sfi2fo were used to amplify the gene coding for the domains (D1 and D2) of non-cysteine pIII; termed as '**non-cys insert**'. The forward primer prepcrba was used to add a linker (Gly-Ser-Gly), while the primer sfi2fo was used to clone a *Sfi1* restriction site. In a second PCR reaction, a '**library insert**' was constructed with the DNA encoding a customized random peptide sequences (GAE$\underline{X_1}\underline{X_2}$LKCLE$\underline{X_3}\underline{X_4}$LKAG; X_n represents any of the 20 natural amino acids). The library insert was cloned with the forward primer sfixxcxx3lba and the reverse primer sfi2fo, using the **non-cys insert** as a template.

After the ligation of 10.0 and 5.0 μg of *the SfiI* digested fdg3p0ss21-stop plasmids and PCR products, the fdg3p0ss21-library vector was electroporated into TG1 *E.coli* competent cells and grown in 2YT/chloramphenicol (30 μg/mL) medium. The size of the phage library was estimated as 2.5×10^6 by counting the colonies on LB/chloramphenicol (30 μg/mL) agar plates. The library diversity was analyzed based on the sequence analysis (with the primer fdg3p0ss21-rev) and sufficient for screening. Quality of the initial phage library was analyzed by sequencing 15 single clones, of which all clones contained the designed peptide sequences. The appearance of amino acids at randomized positions is well consistent with the theoretical appearance, and any mutation is not detected at non-randomized positions. All primer sequences are listed in Table S1, 5` to 3` orientation.

Chemical modification of library peptide on phage

To the phage solution (100 μL, 100-200 nM), 2 μL of 500 μM mannose derivative (Man-Npys) in PBS was added. Man-Npys (Figure S1a) was generously given by Ms. Kanako Arai, which synthesized as previously reported. [S1,S3,S4] The 500 μM Man-Npys solution was prepared by diluting the 50 mM stock 100-fold in PBS, in which the 50 mM stock was pre-diluted with methanol. After incubation at 37^{o}C for 1.0 hr, excess mannose derivatives were removed via dialysis (MWCO 15000; Tube-O-Dialyzer, G-Biosciences) in 100 mL of CA buffer (10 mM HEPES, 150 mM NaCl, 1.0 mM $CaCl_2$, 1.0 $MgCl_2$, 1.0 mM $MnCl_2$, pH7.0, autoclaved), twice for 1.0 hr each session. After inoculation of TG1 cells with mannose-modified and non-modified phages, the titers of phage were estimated as the colony-forming unit (cfu).

Biopanning

Phage particles (10^9 cfu in CA buffer; 100 μL) were mixed with 100 μL of 10 μg/mL biotin-concanavalin A (1.0 μg) and incubated for 1.0 hr at room temperature. After that, mixtures were mixed with 100 μL of streptavidin-coupled magnetic beads (10 mg/mL; pre-washed twice with 500 μL of PBS), to capture the phage-ConA complexes, where the reaction was incubated at room temperature for 15 mins with gentle rotation (<5 rpm). After 5 washes in CAT buffer (CA buffer with 0.1% (v/v) Tween20), the bound phages were eluted with 200 mM Me-Man in CA buffer (15 minutes, room temperature). The eluates were transferred to a new tube and 100 μL of 200 mM Me-Man was again added, to remove completely any bound phages on the beads. The eluated phage stock (200 μL) was used directly to infect 30 mL of log-phase

E.coli TG1 cells (OD_{600} of 0.4-0.5), for 90 min at 37°C. Phage stock was amplified for subsequent rounds of selection.

Phage ELISA

To identify phage clones with high binding to ConA, ELISA was performed as described previously.[S1] Briefly, a 96-well Corning microplate was coated overnight with 100 μL/well of ConA (1μg; 10 μg/mL in CA buffer). After 3 washes with CA buffer, each well was blocked with 100 μL of 1% BSA (w/v) in CA buffer for 1.0 hr at 4°C. Phage clones were diluted to a final concentration of 500 pM, with CA buffer alone or together with Me-Man (1.0 or 100 mM), and added to the corresponding wells (50 μL each, room temperature). After 3 washes with CA buffer, an anti-M13 phage antibody horseradish peroxidase was added at 70 μL/well, at a dilution of 1:5000 in 1% (w/v) BSA-CA buffer and incubated at room temperature for 1.0 hr. The wells were then washed 3 times with CA buffer, and the substrate for fluorogenic enzyme reaction (QuantaBlu Fluorogenic Peroxidase Substrate Kit, Pierce) added. The fluorescence ($\lambda_{excitation}$ = 390nm, $\lambda_{emission}$ = 475nm) was measured with Twinkle microplate fluorometer (Twinkle LB 970, Berthold Technologies).

Peptide synthesis

The candidate peptides were synthesized on a TentaGel S RAM resin (0.24 mmol/g) using the standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry-based strategy. [S5] Fmoc-protected amino acids (3.0 eq.) were coupled for 30 mins, using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 3.0 eq.), 1-hydroxybenzotriazole monohydrate (HOBt□H_2O, 3.0 eq.) and N,N-diisopropylethylamine (DIPEA, 6.0 eq.) in N-methylpyrrolidone (NMP). Removal of

Fmoc groups was performed with 20% (v/v) piperidine in NMP for 15 mins.

The protected peptides were then cleaved from the resin with 9.5 mL of TFA in the presence of 0.25 mL of triisopropylsilane and 0.25 mL distilled water by stirring for 1.0 h. After filtration, the reaction solution was concentrated under reduced pressure, and crude peptides were precipitated in cooled diethyl ether. Crude peptides were purified by RP-HPLC (column, COSMOSIL 5C18-ARII, ϕ10×250 mm). The HPLC solvents employed were ultrapure water containing 0.1% TFA (solvent A) and acetonitrile containing 0.08% TFA (solvent B). All peptides were obtained as TFA salts after lyophilization. Purified peptides were identified by ESI-MS. p1, *m/z* 884.5, calcd 885.1 $[M+2H]^{2+}$, *m/z* 589.9, calcd 590.4 $[M+3H]^{3+}$; p2, *m/z* 862.9, calcd 863.5 $[M+2H]^{2+}$, *m/z* 575.8, calcd 576.0 $[M+3H]^{3+}$; p3, *m/z* 890.5, calcd 890.6 $[M+2H]^{2+}$, *m/z* 593.6, calcd 594.1 $[M+3H]^{3+}$; p6, *m/z* 872.2, calcd 872.5 $[M+2H]^{2+}$, *m/z* 581.6, calcd 582.0 $[M+3H]^{3+}$; p8, *m/z* 823.1, calcd 823.5 $[M+2H]^{2+}$, *m/z* 548.7, calcd 549.3 $[M+3H]^{3+}$; Control, *m/z* 1515.1, calcd 1514.8 $[M+H]^{+}$, *m/z* 758.2, calcd 758.4 $[M+2H]^{2+}$.

Mannose-Modification of Peptides

The purified peptide (2.5-3.0 μmol; 2 mL of 10% acetic acid –water) solution was added drop-wise into a solution of Man-Pys (25.3 μmol; 1.8 mL of methanol; Figure S1b) and the conjugation reaction was allowed to proceed for 1.5 hrs at RT. The solution was concentrated under the reduced pressure, and the excess Man-Pys was removed by gel filtration chromatography using PD-10 Desalting Columns (GE healthcare). Mannose-modified peptides were purified by RP-HPLC and obtained as TFA salts after lyophilization. p1-Man, *m/z* 1003.9, calcd 1004.2 $[M+2H]^{2+}$, *m/z* 669.4,

calcd 669.8 $[M+3H]^{3+}$; p2-Man, *m/z* 982.5, calcd 982.7, $[M+2H]^{2+}$, *m/z* 655.2, calcd 655.4 $[M+3H]^{3+}$; p3-Man, *m/z* 1009.0, calcd 1009.7 $[M+2H]^{2+}$, *m/z* 673.2, calcd 673.5 $[M+3H]^{3+}$; p6-Man, *m/z* 991.1, calcd 991.7, $[M+2H]^{2+}$, *m/z* 661.1, calcd 661.4 $[M+3H]^{3+}$; p8-Man, *m/z* 942.1, calcd 942.6 $[M+2H]^{2+}$, *m/z* 628.4, calcd 628.7 $[M+3H]^{3+}$; Control-Man, *m/z* 877.0, calcd 877.5 $[M+2H]^{2+}$, *m/z* 584.9, calcd 585.4 $[M+3H]^{3+}$.

Secondary structural analysis of peptides by circular dichroism (CD) spectroscopy

All peptides were dissolved in ultrapure water (2.0 mM) and diluted to a final concentration of 50 μM with sodium phosphate buffer (20 mM, pH 7.4) containing 30% of trifluoroethanol (TFE). CD spectra were recorded on a JASCO J-720WI spectropolarimeter using a quartz cell with 0.1 cm path length at 25°C. The α-helical content was estimated by the following equations, from the molecular mean residue ellipticity at 222 nm (Θ_{222}) as previously described [S6,7].

$$\text{Fraction helicity percentage, fH (\%)} = (\Theta_{222} - \Theta_C) / (\Theta_H - \Theta_C) \times 100\%$$

$$100\%\ \alpha\text{-helix}, \Theta_H = (-44000 + 250T)(1 - \chi/N_r)$$

$$100\%\ \text{coil}, \Theta_C = (2220 - 53T)$$

Where Θ_H corresponds to the complete conversion of Θ_{222} for 100% helical while Θ_C estimates the conversion of Θ_{222} for completely coiled forms, T refers to the temperature in °C, χ (= 3) is a constant used to compensate for non-hydrogen bonded carbonyls and N_r is the chain length in residues.

Binding analysis of peptides and concanavalin A by surface plasmon resonance (SPR)

The binding kinetics of synthesized peptides was measured using a SPR system (Biacore X100, GE healthcare). ConA was immobilized to CM5 sensor chip

by amine coupling reaction. Different concentrations of peptide solution (in CA buffer) were injected, where the temperature and flow rate were controlled at 25^{o}C and 30 μL/min. For unmodified ligands, peptides were dissolved in CA running buffer containing 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Association and dissociation reactions were monitored for 120 sec and 240 sec, respectively. Sensorgram was a plot of response against time, and the kinetics were analyzed using BIAevaluation software version 4.1 (assuming a 1:1 Langmuir binding model). The estimation of the equilibrium dissociation constant (K_d) was calculated based on the following equation: $K_d = k_{-1} / k_1$ (M), where k_{-1} *and* k_1 are the kinetic association and dissociation rate constants. The Gibbs free energy (ΔG) of peptides were calculated from the measured K_d, with the equation $\Delta G = RT \ln K_d$ (where T is the absolute temperature [298.15K] and R is the universal gas constant [8.314 $JK^{-1}mol^{-1}$]).[S8]

Binding analysis of Me-Man to ConA by isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) was performed on a MicroCal VP-ITC calorimeter (GE Healthcare). The measurement was conducted at 25^{o}C. Titrations were performed by injecting 20 aliquots of 10 μL of Me-Man/CA buffer solution (2.0 mM) into the ConA/CA buffer solution (15 μM) every 5 min. The heat flow resulting from the binding of Me-Man to ConA was recorded as a function of time and converted into enthalpies (ΔH) by integration of the appropriate reaction peaks (Figure S4). Dilution effects were corrected by subtracting the results of a blank experiment with CA buffer in place of Me-Man/CA buffer solution under identical experimental conditions. ITC data was fitted to a one-to-one binding model to determine the binding parameters

(K_a, ΔH, ΔS), using software Origin (GE Healthcare). The thermodynamic parameters determined for the interaction of Me-Man to ConA yielded K_d = 158.2 μM; ΔH and $T\Delta S$ of -0.31 kcal mol^{-1} and -25.38 kcal mol^{-1}, respectively (Figure S4). The estimation of equilibrium dissociation constant (K_d) was calculated based on the following equation: $K_d = 1 / K_a$.

Sequence of primers

Table S1. PCR primer sequences used for PCR cloning of fdg3p0ss21-library vector, library insert, stop insert, fd0D12 and fdg3p0ss21-stop vector. The *SfiI* restriction enzyme recognition site is underlined. The DNA encoding the semi-random library sequences are shaded and the randomized positions are coded by NNK codon (N= A, T, C, G; K= G or T nucleotide). The usage of NNK codons eliminates two out of three possible stop codons.

Primer name	**DNA sequence (5' to 3')**
pelbsfiecofo	GCATGAATTCCGATGACTGAGGCCGGCTGGGCCGCATAGAAAGGAACAACTAAAGGAAT
ecoG3pNba	GCATGAATTCCAGTCAGTACGGCCTCGGGGGCCATGGCTTCTGGTACCCCGGTTAAC
sfistopba	TATGCGGCCCAGCCGGCCATGGTAATGAGGCGGATCCGGCGCTG
prepcrba	GGCGGATCCGGCGCTGAAACTGTTGAAAGTAG
sfi2fo	GAAGCCATGGCCCCCGAGGCCCCGGACGGAGCATTGACAGG
sfixx**c**xx3lba	TATGCGGCCCAGCCGGCCATGGCAGGCGCGGAANNKNNKCTGAAATGCCTGGAANNKNN KCTGAAAGCGGGCGGATCCGGCGCTG
fdg3p0ss21-rev	TAATTGCTCGACCTCCTCTC

Table S2. The amino acid sequences of identified phage clones. Amino acids with the randomized positions of the synthetic library were underlined. The asterisk (*) represents the single point mutation within the synthetic library (c1clone; a leucine to arginine residue).

Clone	Sequence	Frequency
c1	GAE**FY**R*KCLE**AL**LKAG	65/76
c2	GAE**FY**LKCLE**AL**LKAG	5/76
c3	GAE**TY**LKCLE**RK**LKAG	1/76
c4	GAE**YP**LKCLE**KT**LKAG	1/76
c5	GAE**AQ**LKCLE**DL**LKAG	1/76
c6	GAE**NN**LKCLE**KR**LKAG	1/76
c7	GAE**QN**LKCLE**VF**LKAG	1/76
c8	GAE**SK**LKCLE**AQ**LKAG	1/76

Table S3. The relationship between the dissociation constant (K_d) and the Gibbs Free Energy (ΔG) of Me-Man, p3, and p3-Man peptides.

Ligands	K_d / μM	ΔG / kJ mol^{-1}
Me-Man	158.6	-21.7
p3	21.4	-26.6
p3-Man	1.2	-33.2

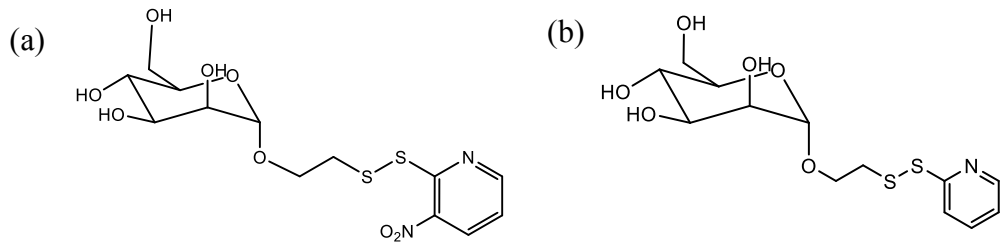

Figure S1. Chemical structure of mannose derivatives; (a) Man-Npys and (b) Man-Pys.[S2] Man-Npys (a) was used for chemical modification on phages, while Man-Pys (b) was used to modify chemically-synthesized peptides.

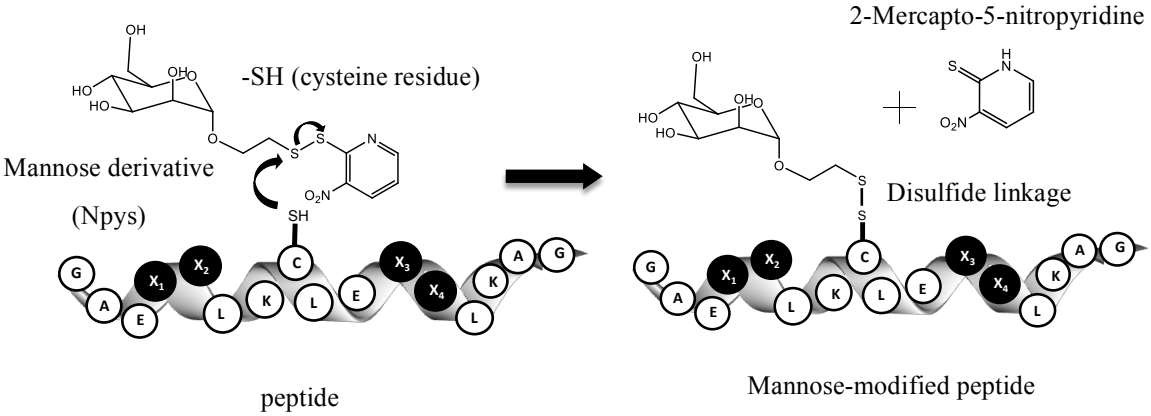

Figure S2. Reaction scheme for the generation a mannose-modified peptide via the thiol group of the cysteine residue, with a 2-mercaptoethyl-α-D-mannose derivative activated by a 3-nitro-2pyridine thiol unit. Pyridyl disulfide react steadily with sulfhydryl group to form of a disulfide bond.

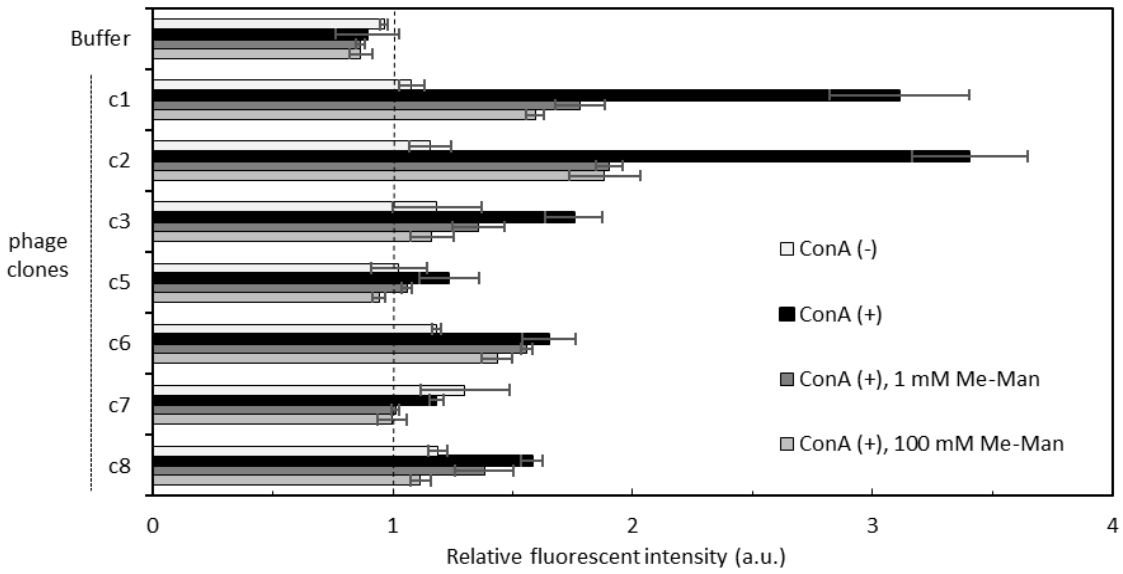

Figure S3. The competitive phage ELISA experiment for the binding of phage clones to immobilized ConA using Me-Man. The black and white bars indicate the relative fluorescence intensity due to ConA immobilized and non-immobilized microplates, respectively. The light and dark gray bars represent the relative fluorescence intensity in the presence of 100 mM and 1.0 mM of Me-Man. ConA was immobilized with the concentration of 10 μg/mL (0.38 μM) and phage concentrations were 0.5 nM. For all samples, n = 3. Error bars represent the standard deviation.

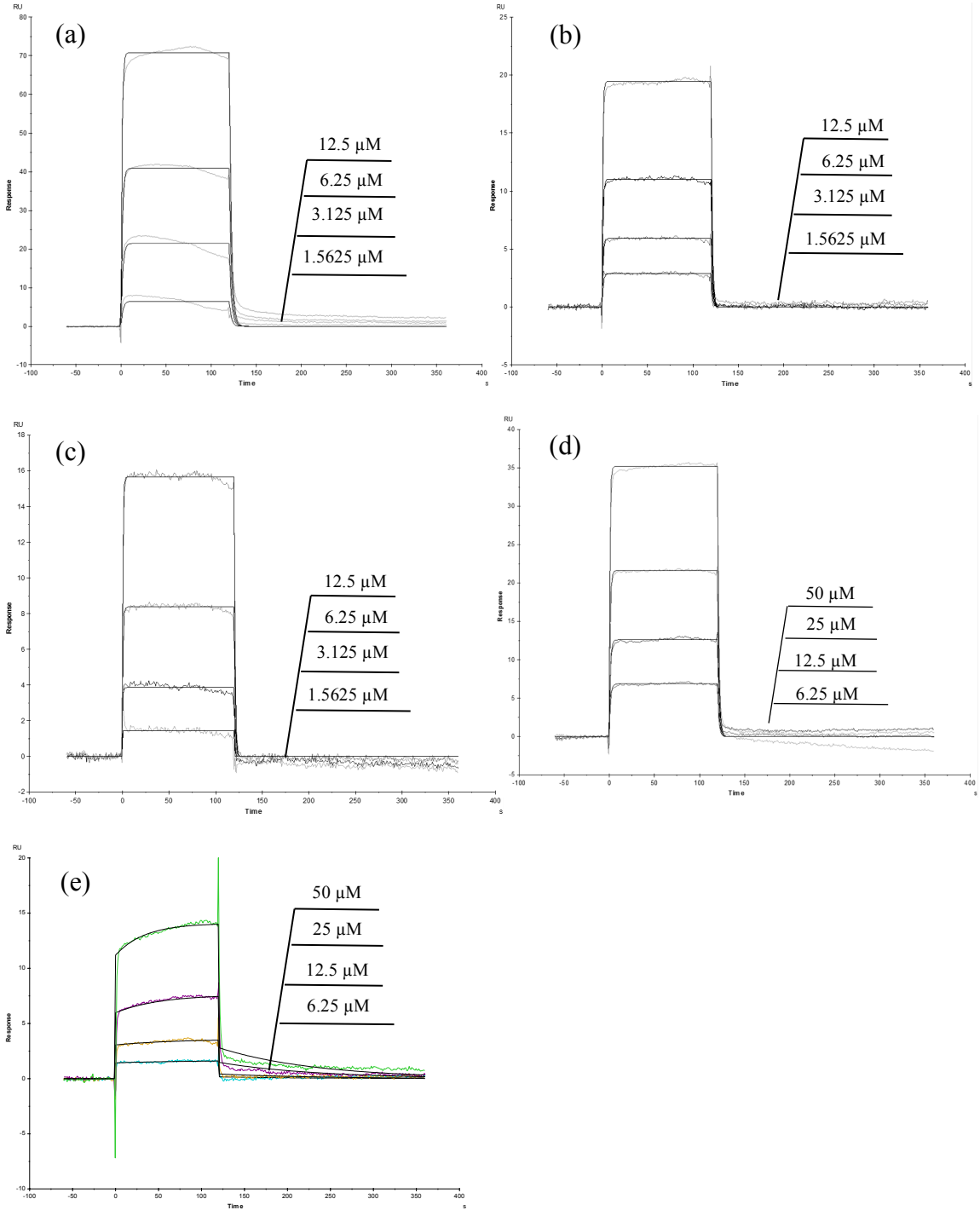

Figure S4. SPR sensorgrams of (a) p1-Man, (b) p6-Man, (c) p8-Man, (d) Control-Man, and (e) p3 peptides. Peptide concentrations are shown in the figure. Gray curves represent fitting curves assuming a 1:1 Langmuir binding model.

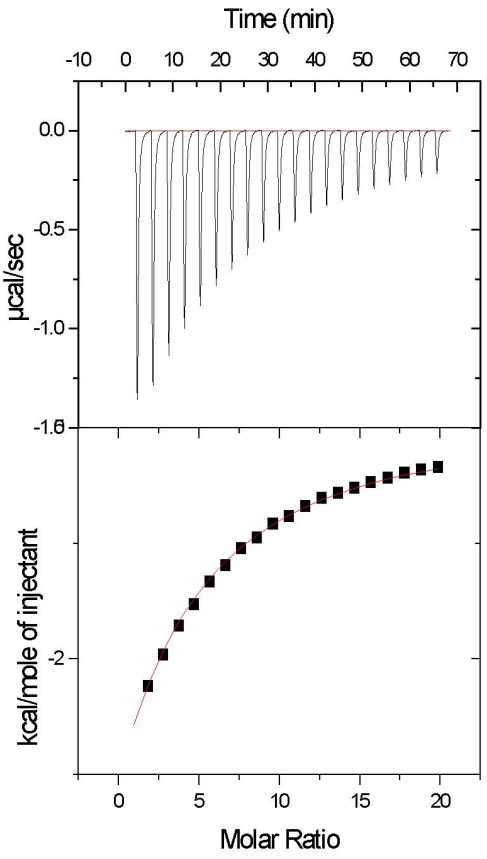

Figure S5. ITC titration curves and processed date of Me-Man to ConA. Measurement conditions: [ConA] = 15 µM, [Me-Man] = 2.0 mM (10 µL, 20 injections), at 25◦C in CA buffer (pH 7.0).

References

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