

**Supporting Information for**  
**Metal Sequestration and Antimicrobial Activity of Human Calprotectin are pH-Dependent**

Tomer Rosen and Elizabeth M. Nolan\*

Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue,  
Cambridge, MA 02139, USA

\*Corresponding author: [lnolan@mit.edu](mailto:lnolan@mit.edu)

Phone: 617-452-2495

This Supporting Information includes:

<b>Complete Experimental Section</b> .....	S4
General materials and methods .....	S4
General protein methods.....	S4
Circular dichroism spectroscopy .....	S4
Analytical size exclusion chromatography.....	S5
Antimicrobial activity assays .....	S6
Metal depletion assay.....	S7
Mn(II) binding monitored by analytical SEC/ICP-MS.....	S7
Determination of apparent Mn(II) dissociation constant ( $K_{d,Mn(II)}$ ) of ZP1 at low pH .....	S8
Mn(II) competition titration with ZP1.....	S9
Low-temperature EPR spectroscopy of Mn(II)-CP.....	S9
Mn(II) competition assay monitored by B-CP pull-down.....	S10
<b>Supporting Tables</b> .....	S11
<b>Table S1.</b> Proteins employed in this study.....	S11
<b>Table S2.</b> Bacterial and fungal strains used in this study .....	S11
<b>Table S3.</b> pH of bacterial cultures in MES-buffered growth media .....	S12
<b>Table S4.</b> Analytical SEC experiments of CP-Ser with Ca(II).....	S13
<b>Table S5.</b> Metal analysis of MES:TSB medium treated with CP-Ser.....	S14
<b>Table S6.</b> Metal analysis of MES:LB medium treated with CP-Ser.....	S15
<b>Table S7.</b> Analytical SEC experiments of CP-Ser with Mn(II).....	S16
<b>Supporting Figures</b> .....	S17
<b>Figure S1.</b> CD spectra of CP-Ser.....	S17
<b>Figure S2.</b> CD spectra of CP .....	S18
<b>Figure S3.</b> Thermal denaturation of CP-Ser .....	S19
<b>Figure S4.</b> Melting temperature of CP-Ser as a function of pH .....	S20
<b>Figure S5.</b> Analytical SEC chromatograms of CP-Ser with Ca(II) .....	S21
<b>Figure S6.</b> Antimicrobial activity of CP-Ser (20 h).....	S22
<b>Figure S7.</b> Antimicrobial activity of CP (8 h).....	S23
<b>Figure S8.</b> Antimicrobial activity of CP (20 h).....	S24
<b>Figure S9.</b> Metal analysis of MES:TSB medium treated with CP-Ser.....	S25
<b>Figure S10.</b> Metal analysis of MES:LB medium treated with CP-Ser .....	S26
<b>Figure S11.</b> Mn(II) retention by CP-Ser following SEC .....	S27

<b>Figure S12.</b> ZP1 $K_{d,Mn(II)}$ determination .....	S28
<b>Figure S13.</b> Mn(II) competition assay between B-CP and MntC/PsaA .....	S29
<b>Figure S14.</b> SDS-PAGE from Mn(II) competition assay between B-CP and MntC/PsaA....	S30
<b>Figure S15.</b> Mn(II) retention by MntC/PsaA following SEC .....	S30
<b>Supplementary References</b> .....	S31

## Experimental Methods

**General materials and methods.** All solvents, reagents, and chemicals were obtained from commercial suppliers and used as received. All solutions were prepared using Milli-Q water (18.2 M $\Omega$ -cm, 0.22- $\mu$ m filter, EMD Millipore) and buffers were sterile filtered (0.2  $\mu$ m) before use. 2-(*N*-morpholino)ethanesulfonic acid (MES) (ULTROL, free acid, Calbiochem) and NaCl (Suprapur, EMD Millipore) were used to prepare buffers for metal competition experiments and EPR spectroscopy. HCl solution (TraceSELECT, >30%, Fluka) and aqueous NaOH solution (Suprapur, 30%, EMD Millipore) were used for pH adjustment. Buffers for all other experiments were prepared using high purity NaCl (BioXtra, Sigma) and NaOH (proteomics grade, VWR). All buffers were stored in polystyrene bottles (Corning). Stock solutions of Ca(II) (1 M, 500 mL) and Mn(II) (100 mM, 10 mL) were prepared by dissolving CaCl<sub>2</sub>·2H<sub>2</sub>O (BioUltra or 99.99% trace metal, Sigma) and MnCl<sub>2</sub>·4H<sub>2</sub>O (99.99% trace metal, Sigma), respectively, in Milli-Q water. The metal stock solutions were prepared in nitric acid-washed volumetric glassware and stored in sterile polypropylene tubes.

**General protein methods.** The human CP heterodimer and its variants,<sup>1</sup> staphylococcal MntC and streptococcal PsaA<sup>2</sup> (Table S1) were recombinantly expressed, purified, and characterized as described previously. The purified proteins were stored as aliquots at -80 °C, and only thawed once immediately before use. Protein concentrations were determined by optical absorbance at 280 nm using a BioTek Synergy HT plate reader outfitted with a calibrated Take3 Micro-Volume plate. Calculated extinction coefficients (<https://web.expasy.org/protparam/>) used for the determination of protein concentration are listed in Table S1. All experiments were performed with protein originating from two different expression and purification processes.

**Circular dichroism spectroscopy.** A Jasco J-1500 circular dichroism (CD) spectrometer thermostatted at 25 °C was used for CD spectroscopy. A 1-mm path-length CD cell (Hellma) was employed for all CD measurements. Protein samples were buffer-exchanged (4x) into CD buffer

(10 mM MES, pH 5.0–7.0) using a 10-kDa MWCO spin filter (Amicon, EMD Millipore) and diluted with CD buffer to a final concentration of 10  $\mu$ M. For samples that contained Ca(II), an aliquot from a 1 M Ca(II) stock solution was added to the protein solution to afford a final Ca(II) concentration of 2 mM, and the sample was incubated at room temperature for 30 min prior to data collection. The CD spectra were recorded from 195 to 260 nm using continuous scan mode (50 nm/min) and 1 nm bandwidth. The averages from three scans for each condition are presented. The baseline was subtracted from a sample of CD buffer. For thermal denaturation experiments, the temperature was increased from 25 to 95 °C in 1 °C intervals (1 deg/min heating rate), and the CD intensity was recorded at 222 nm. Each thermal denaturation experiment was repeated on two independent samples, and data from one representative experiment are shown.

**Analytical size exclusion chromatography.** An ÄKTA purifier with a Superdex 75 10/300 GL column (GE Healthcare Life Sciences) housed at 4 °C was used to perform analytical size exclusion chromatography (SEC) experiments. The running buffer was 75 mM MES, 100 mM NaCl, pH 5.0–7.0. The pH of the buffer was adjusted with NaOH solution at ambient temperature. The column was calibrated at each pH with blue dextran and a low-molecular-weight calibration mixture (GE Healthcare Life Sciences) consisting of aprotinin (6.5 kDa), ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa) and ovalbumin (44 kDa) prior to use. Protein was thawed at room temperature and buffer exchanged (4x) into the running buffer using a 10-kDa MWCO spin filter. Samples (100  $\mu$ M, 300  $\mu$ L) were prepared and loaded into a 100  $\mu$ L injection loop, and the FPLC system was programmed to inject 500  $\mu$ L of sample onto the column. The samples were eluted over one column volume (24 mL) at a flow rate of 0.5 mL/min. For experiments with Ca(II), 2, 5, 10 or 20 mM Ca(II) was added from a 1 M stock solution to both the running buffer and sample. The column was pre-equilibrated with the appropriate Ca(II) containing running buffer before each experiment. At least two independent replicates were performed for each experiment, and representative data from one experiment are shown.

**Antimicrobial activity assays.** Antimicrobial activity (AMA) assays were performed by adapting reported protocols.<sup>1</sup> Protein aliquots were buffer-exchanged (4x) into MES buffer (100 mM MES, 100 mM NaCl, pH 5.0–7.0) using a 10-kDa MWCO spin filter, and further diluted in MES buffer to afford 10x stock solutions (5 mg/mL or 10 mg/mL). AMA media were prepared by combining a 62:38 v/v ratio of MES buffer (100 mM MES, 100 mM NaCl, pH 5.0–7.0) and TSB growth medium, and supplementing the medium with 2 mM Ca(II). The pH of the media was readjusted to 5.0–7.0 with aqueous NaOH or HCl, and the pH-adjusted media were sterile filtered (0.22- $\mu$ m filter system, Corning) into polystyrene bottles. Bacteria (Table S2) were stored as freezer stocks at -80 °C, streaked on TSB 1.5% agar plates and incubated at 37 °C (16 h). A single colony was used to inoculate 3 mL TSB and the culture was incubated at 37 °C (16 h, 150 rpm). The overnight cultures were diluted 1:100 into 2 mL of TSB and grown at 37 °C (150 rpm) until the OD<sub>600</sub> reached  $\approx$  0.6 (t  $\approx$  2–3 h). Each culture was then diluted 1:500 into the appropriate AMA medium, and the assays were immediately set up in 96-well plates (Costar, TC-treated, Corning) using 90  $\mu$ L of the diluted bacterial culture and 10  $\mu$ L of the protein 10x stocks (or MES buffer for a no protein control). Each condition was performed in triplicate in the assay plate. The plates were wrapped in moist paper towels and plastic wrap and incubated with shaking at 150 rpm at 37 °C. The OD<sub>600</sub> was measured at 8 and 20 h using a BioTek Synergy HT plate reader. At least three independent replicates of each assay were performed, and the resulting averages and standard deviation are reported. The pH of the cultures after 8 and 20 h of incubation (37 °C, 150 rpm) was measured in a separate experiment under the conditions described above (Table S3) using a portable pH meter (Hanna instruments) equipped with an extended length pH electrode.

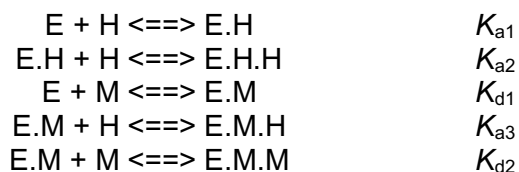
**Metal depletion assay.** The assay was adapted from a reported procedure.<sup>3</sup> Protein aliquots were buffer-exchanged (4x) into MES buffer (20 mM MES, 100 mM NaCl, pH 5.0–7.0) using a 10-kDa MWCO spin filter. AMA media were prepared as described above, except that 20 mM MES, 100 mM NaCl aqueous buffer was used. Aliquots (1-mL) of AMA medium were transferred to sterile polypropylene microcentrifuge tubes (1.7 mL, VWR). Protein was then added from a concentrated stock solution to afford a final concentration of 250  $\mu\text{g/mL}$ . Untreated AMA media and CP-treated media samples were incubated for 20 h (30 °C, 150 rpm). The samples were spin-filtered (10-kDa MWCO 4-mL Amicon tubes, EMD Millipore) and the filtrate was collected. Samples for ICP-MS analyses were prepared by combining 700  $\mu\text{L}$  of the filtrate with 700  $\mu\text{L}$  of ~3% nitric acid (TraceSELECT, Fluka) and spiking the resulting mixture with 20  $\mu\text{L}$  (10 ppb) of terbium internal standard (Agilent). Metal ion concentrations were quantified using an Agilent 7900 ICP-MS instrument operated in helium mode. Calibration was performed prior to each analysis with a series of five serially diluted (1:10 in ~3% nitric acid) samples of the Environmental Calibration Standard (Agilent) as well as a nitric acid-only standard. Each standard and sample was spiked with Tb internal standard as described above.

**Mn(II) binding monitored by analytical SEC/ICP-MS.** Analytical SEC was performed as described above. Protein samples (200  $\mu\text{M}$ , 300  $\mu\text{L}$ ) were incubated with 10 equivalents of Mn(II) (added from a 100 mM Mn(II) stock solution in Milli-Q water) for >30 min at 4 °C. Fractions (0.5 mL) were collected in polypropylene tubes (VWR). For experiments with Ca(II), 4 mM Ca(II) was added from a 1 M stock solution to both the running buffer and sample. The sample was incubated with Ca(II) for >20 min at 4 °C prior to Mn(II) addition. The protein concentration of each collected fraction was determined by absorbance at 280 nm in acid-washed quartz cuvettes using a Beckman DU 800 UV–Vis spectrophotometer thermostatted at 25 °C with a Peltier temperature controller. The Mn concentration of each collected fraction was determined by ICP-MS as described above. ICP-MS samples were prepared by combining 400  $\mu\text{L}$  of analytical SEC fraction

with 1000  $\mu\text{L}$  of  $\sim 3\%$  nitric acid and 20  $\mu\text{L}$  (10 ppb) of Tb internal standard. Two independent replicates were performed for each experiment, and representative data from one experiment are shown.

#### Determination of apparent Mn(II) dissociation constant ( $K_{d,\text{Mn(II)}}$ ) of ZP1 at low pH.

The fluorescent probe Zinpyr-1 (ZP1, stored as aliquots in DMSO at  $-20\text{ }^\circ\text{C}$ ) was added to 2 mL of titration buffer (75 mM MES, 100 mM NaCl, pH 5.5–7.0) in a 1-cm path length nitric acid washed quartz cuvette to afford a final concentration of 5  $\mu\text{M}$ . Each solution was titrated with Mn(II) (2  $\mu\text{L}$  aliquots of a 500  $\mu\text{M}$  Mn(II) solution in titration buffer). After each Mn(II) addition, the solution was gently mixed and incubated for  $>10$  min at room temperature in the dark, and then the fluorescence emission spectrum was recorded. Emission spectra were collected on a Photon Technologies International QuantaMaster 40 fluorimeter outfitted with a continuous xenon source for excitation, auto calibrated QuadraScopic monochromators, a multimode PMT detector, and a circulating water bath maintained at  $25\text{ }^\circ\text{C}$ . This instrument was controlled by the FelixGX software package. The excitation wavelength was 490 nm (0.4 mm excitation and emission slit widths). The emission spectra were collected and integrated over 500–650 nm. The relative integrated emission of each solution was plotted against the added Mn(II) equivalents, and the data were fitted to a two-site binding model by nonlinear least-squares regression using DynaFit.<sup>4</sup> ZP1 has two di-(2-picolyl)amine-based ligands for metal complexation. Protonation equilibria of ZP1 were included in the model as described previously,<sup>5</sup> resulting in the following equilibria:



Where “E” is ZP1, “M” is a Mn(II) ion, and “H” is a proton.  $pK_a$  values of ZP1 were previously reported.<sup>6</sup> Both  $K_{d1,\text{Mn(II)}}$  and  $K_{d2,\text{Mn(II)}}$  are reported in Figure S12. Only the  $K_{d1,\text{Mn(II)}}$  values are given



in the main text. These values represent the high affinity Mn(II) site of ZP1 and are used to inform the Mn(II) affinity of CP-Ser at each pH value.

**Mn(II) competition titration with ZP1.** Mn(II) competition titrations between ZP1 and CP-Ser were performed as described previously.<sup>7</sup> The protein was buffer-exchanged (4x) using a 10-kDa MWCO spin filter into MES buffer (75 mM MES, 100 mM NaCl, pH 5.5–7.0). For each titration, a 2 mL solution of titration buffer containing ZP1 (1  $\mu$ M) and CP-Ser (4  $\mu$ M) was prepared in a 1-cm path length nitric acid washed quartz cuvette. For experiments with Ca(II), 200  $\mu$ M of Ca(II) was added from a 100 mM Ca(II) solution to the titration buffer, and the sample was incubated for ~20 min at room temperature. The mixture was titrated with Mn(II) (2 or 4  $\mu$ L of a 500  $\mu$ M Mn(II) solution in titration buffer). After each Mn(II) addition, the solution was gently mixed and incubated for >10 min at room temperature in the dark, after which the fluorescence emission was recorded as described above.

**Low-temperature EPR spectroscopy of Mn(II)-CP.** CP-Ser was buffer-exchanged (4x) into MES buffer (75 mM MES, 100 mM NaCl, pH 5.5–7.0) as described above. Protein samples (200  $\mu$ M, 300  $\mu$ L) were incubated with 0.9 equivalents of Mn(II) (added from 1 mM Mn(II) stock solution in Milli-Q water) for 20 min at room temperature. For experiments with Ca(II), 2 mM Ca(II) was added from a 1 M stock solution and the sample was incubated for 20 min at room temperature prior to Mn(II) addition. The samples were transferred to a 4-mm (OD) quartz EPR tubes (Wilmad) using a Gastight syringe (Hamilton) and slowly frozen in liquid nitrogen. Low-temperature continuous-wave (CW) electron paramagnetic resonance (EPR) spectra were collected on a Bruker EMX-Plus spectrometer with an ER4119HS high sensitivity X-band resonator and a Bruker/ColdEdge 4K waveguide cryogen-free cryostat housed in the MIT Department of Chemistry Instrumentation Facility. Instrument parameters: temperature, 10 K; microwaves, 0.2 mW at 9.38 GHz; modulation amplitude, 1.0 mT at 100 kHz.

**Mn(II) competition assay monitored by B-CP pull-down.** Pull-down experiments were performed by adapting a reported protocol.<sup>2</sup> Biotinylated CP (B-CP) was prepared as previously described<sup>2, 8</sup> and buffer exchanged (4x) into MES buffer (75 mM MES, 100 mM NaCl, pH 5.5–7.0) as described above. The SBPs MntC or PsaA were buffer-exchanged extensively (8x) into MES buffer to remove residual EDTA that was added during the protein purification steps.<sup>2</sup> Samples (600  $\mu$ L) containing 20  $\mu$ M MntC or PsaA, 20  $\mu$ M B-CP, and 18  $\mu$ M Mn(II) were prepared in plastic microcentrifuge tubes (1.7 mL, VWR). Mn(II) was either added last or pre-incubated for 30 min at room temperature with either CP-Ser or a SBP. The samples were incubated for 16 h at room temperature on a tube rotator, followed by B-CP pull-down with streptavidin resin. Immediately prior to use, streptavidin agarose resin (Novagen, EMD Millipore, 350  $\mu$ L of 50% suspension) was transferred to a Spin-X centrifuge tube with a 0.22- $\mu$ m cellulose acetate membrane (Corning) and washed (3x) with MES buffer at the appropriate pH. An aliquot (300  $\mu$ L) from each sample was transferred to the Spin-X tube and incubated with the washed resin for 45 min at room temperature. The samples were then centrifuged (3 min, 13,000 rpm, 20 °C) to separate the B-CP-containing resin and SBP-containing flow-through. Samples before and after B-CP pull-down were analyzed by SDS-PAGE. ICP-MS samples were prepared by combining 200  $\mu$ L of the mixtures before pull-down or flow-through with 1200  $\mu$ L of ~3% nitric acid and 20  $\mu$ L (10 ppb) of Tb internal standard.

## Supporting Tables

**Table S1.** Proteins employed in this study.

Protein	Description	MW (kDa) <sup>a</sup>	$\epsilon_{280}$ (M <sup>-1</sup> cm <sup>-1</sup> ) <sup>b</sup>	Ref.
CP	Human S100A8/S100A9	24.0	18,450	1
CP-Ser	Cys→Ser variant, S100A8(C42S)/S100A9(C3S)	24.0	18,450	1
B-CP	Biotinylated CP, S100A8(C42S)/S100A9(C3-biotin polyethyleneoxide)	24.3	18,450	2
MntC	MntC from <i>S. aureus</i> USA300 JE2 <sup>c</sup>	32.8	35,870	2
PsaA	PsaA from <i>S. pneumoniae</i> D39 <sup>c</sup>	32.4	35,870	2

<sup>a</sup> Molecular weights were calculated by using the ProtParam tool available on the ExPASy server (<http://web.expasy.org/protparam>). <sup>b</sup> Extinction coefficients (280 nm) were calculated by using the ProtParam tool. <sup>c</sup> Prepared without the N-terminal lipid anchor.

**Table S2.** Bacteria used in this study.

Strain	Growth Medium	Source
<i>Escherichia coli</i> UTI89	TSB	Cegelski lab (Stanford)
<i>Salmonella enterica</i> serovar Typhimurium IR715	TSB	Raffatellu lab (UCSD)
<i>Staphylococcus aureus</i> USA300 JE2	TSB	NARSA repository
<i>Pseudomonas aeruginosa</i> PAO1	TSB	Oglesby-Sherrouse lab (UMD)
<i>Klebsiella pneumoniae</i> ATCC 13883	TSB	ATCC
<i>Acinetobacter baumannii</i> ATCC 17961	TSB	ATCC

**Table S3.** pH of bacterial cultures in MES-buffered growth medium.<sup>a</sup>

Strain	AMA medium	Initial pH	pH (8 h) <sup>b</sup>	pH (20 h) <sup>c</sup>
<i>E. coli</i> UTI89	TSB:MES	7.0	7.0	7.0
		6.0	6.1	6.3
		5.0	5.4	5.6
<i>S. Typhimurium</i> IR715	TSB:MES	7.0	7.0	7.0
		6.0	6.2	6.3
		5.0	5.4	5.7
<i>S. aureus</i> JE2	TSB:MES	7.0	6.9	7.1
		6.0	6.1	6.1
		5.0	5.1	5.4
<i>P. aeruginosa</i> PAO1	TSB:MES	7.0	7.1	7.0
		6.0	6.2	6.2
		5.0	5.2	5.5
<i>K. pneumoniae</i> ATCC 13883	TSB:MES	7.0	6.9	7.0
		6.0	6.1	6.2
		5.0	5.3	5.5
<i>A. baumannii</i> ATCC 17961	TSB:MES	7.0	7.0	7.0
		6.0	6.1	6.2
		5.0	5.2	5.5

<sup>a</sup> Overnight cultures (37 °C, 150 rpm) were diluted 1:100 into 2 mL of TSB medium and grown at 37 °C (150 rpm) until the OD<sub>600</sub> reached ≈ 0.6. Each culture was then diluted 1:500 into 5 mL of the appropriate AMA medium, and incubated at 37 °C (150 rpm). Aliquots (0.5 mL) were taken after 8 and 20 h of incubation, and the pH was measured. <sup>b</sup> pH of cultures after 8 h of incubation. <sup>c</sup> pH of cultures after 20 h of incubation.

**Table S4.** Elution volume and calculated molecular weights from analytical SEC.<sup>a</sup>

pH	[Ca(II)] (mM) <sup>b</sup>	Ca(II) equiv.	Elution volume (mL) <sup>c</sup>	Calc. Molecular Weight (KDa) <sup>d</sup>
7.0	0	0	11.5	30.1
7.0	2	20	10.7	35.7
7.0	10	100	10.7	35.8
7.0	20	200	10.7	35.7
6.5	0	0	11.6	30.0
6.5	2	20	10.8	34.9
6.5	10	100	10.7	35.1
6.5	20	200	10.7	35.2
6.0	0	0	11.6	29.6
6.0	2	20	10.9	33.9
6.0	5	50	10.8	34.4
6.0	10	100	10.7	34.7
6.0	20	200	10.7	35.0
5.5	0	0	11.7	29.2
5.5	2	20	11.1	32.8
5.5	5	50	10.9	33.5
5.5	10	100	10.8	34.1
5.5	20	200	10.7	34.7
5.0	0	0	11.9	28.5
5.0	2	20	11.2	32.1
5.0	5	50	11.0	33.2
5.0	10	100	10.9	33.7
5.0	20	200	10.8	34.6

<sup>a</sup> Each sample contained 100  $\mu$ M CP-Ser (75 mM MES, 100 mM NaCl, pH 5.0–7.0). <sup>b</sup> Ca(II) was added to the sample and running buffer. <sup>c</sup> The elution volume corresponds to the maximum peak absorbance at 280 nm. <sup>d</sup> Molecular weights were estimated by the elution volumes of protein standards at pH 5.0–7.0.

**Table S5.** Metal content ( $\mu\text{M}$ ) of MES:TSB medium treated with 250  $\mu\text{g}/\text{mL}$  CP-Ser.<sup>a</sup>

Metal	pH	Untreated <sup>b</sup>	Untreated, fil <sup>c</sup>	CP-Ser <sup>d</sup>	CP-Ser + Ca(II) <sup>e</sup>
Mn	7.0	0.190 (0.009)	0.138 (0.001)	0.003 (0.001)	0.004 (0.001)
	6.0	0.180 (0.004)	0.160 (0.004)	0.009 (0.001)	0.005 (0.003)
	5.0	0.183 (0.004)	0.155 (0.001)	0.178 (0.002)	0.089 (0.006)
Fe	7.0	5.570 (0.110)	1.960 (0.060)	1.000 (0.110)	0.560 (0.070)
	6.0	5.490 (0.020)	2.120 (0.140)	1.140 (0.060)	0.380 (0.010)
	5.0	5.560 (0.040)	2.020 (0.050)	1.980 (0.180)	1.490 (0.120)
Co	7.0	0.036 (0.004)	0.023 (0.002)	0.022 (0.001)	0.023 (0.001)
	6.0	0.037 (0.005)	0.025 (0.002)	0.021 (0.002)	0.022 (0.002)
	5.0	0.036 (0.005)	0.025 (0.002)	0.023 (0.001)	0.025 (0.002)
Ni	7.0	0.254 (0.013)	0.267 (0.017)	0.268 (0.058)	0.322 (0.041)
	6.0	0.256 (0.011)	0.295 (0.023)	0.391 (0.029)	0.365 (0.027)
	5.0	0.258 (0.012)	0.303 (0.030)	0.599 (0.048)	0.299 (0.037)
Cu	7.0	0.131 (0.003)	0.094 (0.004)	0.038 (0.002)	0.033 (0.002)
	6.0	0.146 (0.004)	0.101 (0.003)	0.032 (0.010)	0.009 (0.001)
	5.0	0.129 (0.005)	0.085 (0.003)	0.074 (0.001)	0.058 (0.008)
Zn	7.0	6.570 (0.110)	4.550 (0.080)	0.150 (0.060)	0.090 (0.040)
	6.0	6.540 (0.050)	5.190 (0.060)	0.070 (0.020)	0.070 (0.020)
	5.0	6.630 (0.060)	5.820 (0.070)	0.330 (0.020)	0.140 (0.040)

<sup>a</sup> Metal content was determined by ICP-MS (mean  $\pm$  SEM,  $n \geq 4$ ). <sup>b</sup> untreated medium. <sup>c</sup> untreated medium after spin filtration. <sup>d</sup> medium treated with 250  $\mu\text{g}/\text{mL}$  CP-Ser (10.4  $\mu\text{M}$ ). <sup>e</sup> medium treated with 250  $\mu\text{g}/\text{mL}$  CP-Ser (10.4  $\mu\text{M}$ ) in the presence of 2 mM Ca(II).

**Table S6.** Metal content ( $\mu\text{M}$ ) of MES:LB medium treated with 250  $\mu\text{g}/\text{mL}$  CP-Ser.<sup>a</sup>

Metal	pH	Untreated <sup>b</sup>	Untreated, fil <sup>c</sup>	CP-Ser <sup>d</sup>	CP-Ser + Ca(II) <sup>e</sup>
Mn	7.0	0.120 (0.006)	0.073 (0.005)	0.011 (0.003)	0.007 (0.002)
	6.0	0.123 (0.002)	0.119 (0.004)	0.008 (0.001)	0.006 (0.001)
	5.0	0.125 (0.001)	0.098 (0.007)	0.131 (0.006)	0.050 (0.006)
Fe	7.0	1.771 (0.022)	0.756 (0.036)	0.507 (0.049)	0.204 (0.029)
	6.0	1.730 (0.058)	0.598 (0.042)	0.381 (0.102)	0.344 (0.054)
	5.0	1.763 (0.026)	0.621 (0.016)	0.457 (0.026)	0.334 (0.108)
Co	7.0	0.076 (0.005)	0.068 (0.002)	0.058 (0.002)	0.057 (0.001)
	6.0	0.082 (0.002)	0.067 (0.002)	0.055 (0.001)	0.051 (0.004)
	5.0	0.082 (0.002)	0.070 (0.003)	0.054 (0.001)	0.054 (0.001)
Ni	7.0	0.075 (0.011)	0.298 (0.024)	0.073 (0.018)	0.051 (0.012)
	6.0	0.064 (0.004)	0.191 (0.023)	0.195 (0.029)	0.048 (0.016)
	5.0	0.066 (0.004)	0.242 (0.025)	0.216 (0.027)	0.106 (0.023)
Cu	7.0	0.066 (0.004)	0.051 (0.005)	0.030 (0.006)	0.024 (0.007)
	6.0	0.069 (0.004)	0.043 (0.007)	0.027 (0.007)	0.017 (0.004)
	5.0	0.072 (0.006)	0.049 (0.006)	0.039 (0.008)	0.030 (0.008)
Zn	7.0	6.249 (0.117)	3.476 (0.169)	0.347 (0.086)	0.243 (0.028)
	6.0	6.447 (0.125)	4.075 (0.102)	0.193 (0.018)	0.211 (0.036)
	5.0	6.405 (0.089)	5.258 (0.089)	0.379 (0.028)	0.139 (0.021)

<sup>a</sup> Metal content was determined by ICP-MS (mean  $\pm$  SEM,  $n \geq 4$ ). <sup>b</sup> untreated medium. <sup>c</sup> untreated medium after spin filtration. <sup>d</sup> medium treated with 250  $\mu\text{g}/\text{mL}$  CP-Ser (10.4  $\mu\text{M}$ ). <sup>e</sup> medium treated with 250  $\mu\text{g}/\text{mL}$  CP-Ser (10.4  $\mu\text{M}$ ) in the presence of 2 mM Ca(II).

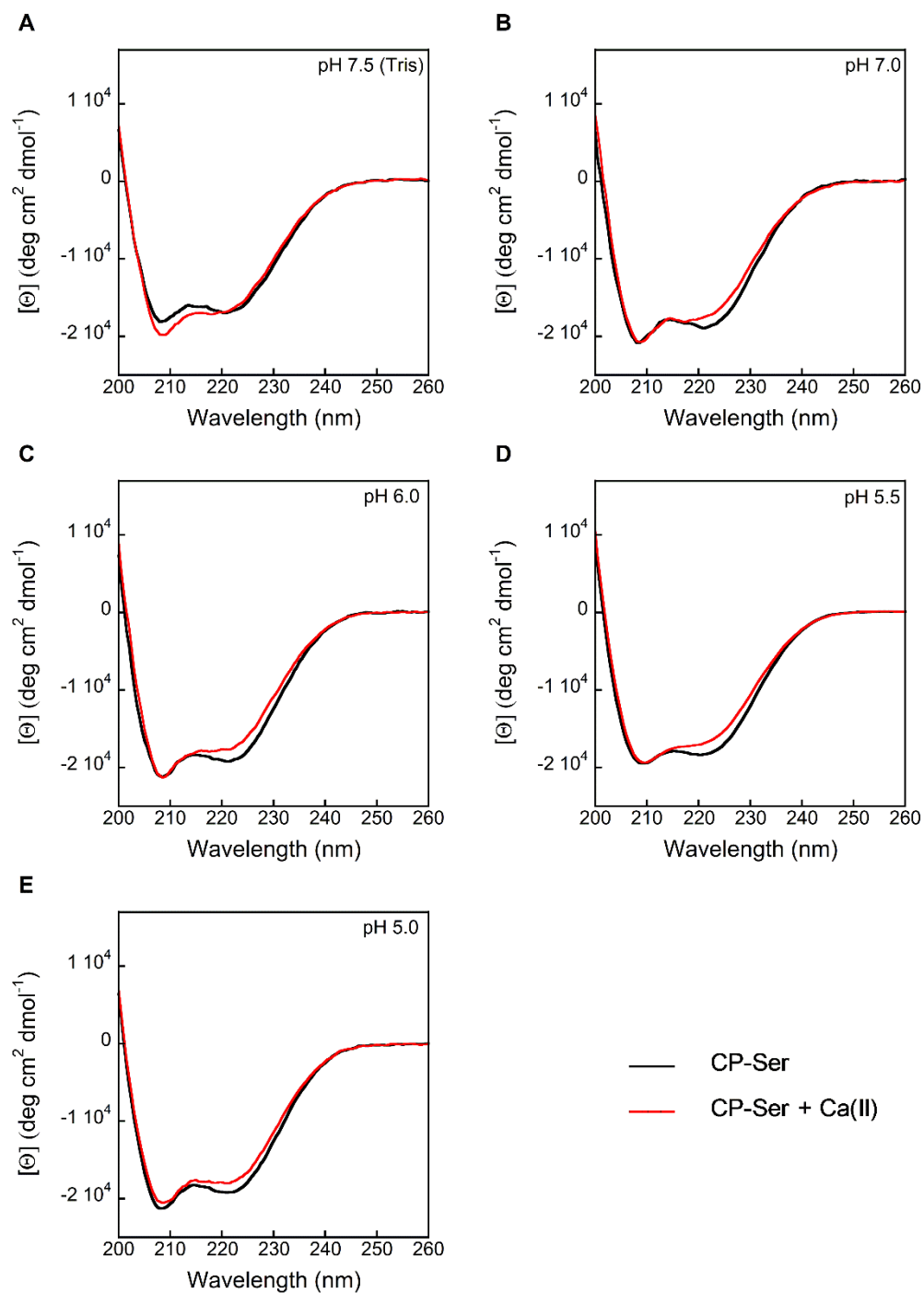
**Table S7.** Elution volume and calculated molecular weights from analytical SEC experiments of Mn(II) binding to CP-Ser.<sup>a</sup>

pH	[Mn(II)] (mM) <sup>b</sup>	[Ca(II)] (mM) <sup>c</sup>	Elution volume (mL) <sup>d</sup>	Calc. Molecular Weight (KDa) <sup>e</sup>
7.0	-	-	11.5	30.1
7.0	2	-	11.1	33.1
6.5	-	-	11.6	30.0
6.5	2	-	11.1	32.7
6.0	-	-	11.6	29.6
6.0	2	-	11.3	32.8
5.5	-	-	11.7	29.2
5.5	2	-	11.7	29.2
5.5	2	4	11.3	31.6
5.0	-	-	11.9	28.5
5.0	2	-	11.7	28.5
5.0	2	4	11.3	31.9

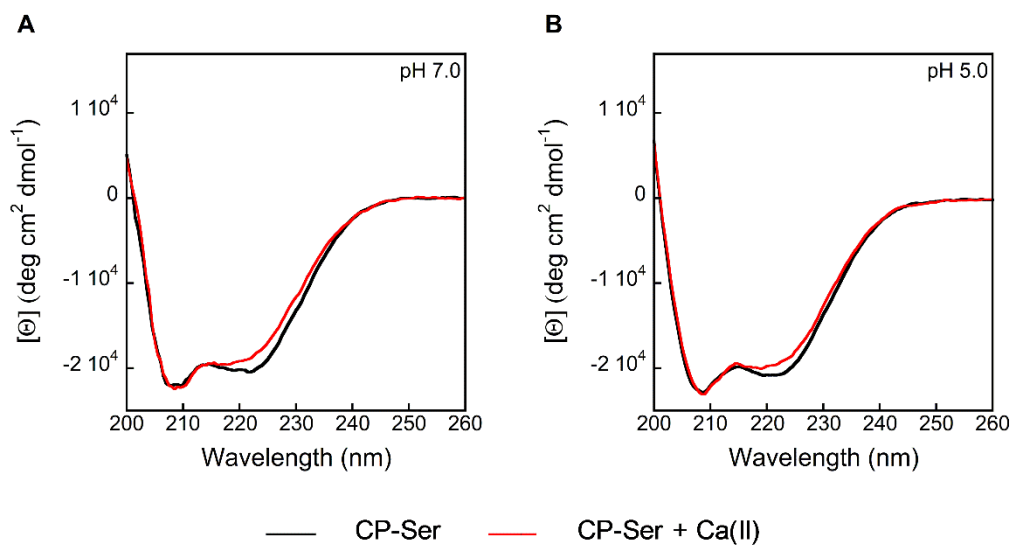
<sup>a</sup> Each sample contained 200  $\mu$ M protein (75 mM MES, 100 mM NaCl, pH 5.0–7.0). <sup>b</sup> Mn(II) was added only to the sample. <sup>c</sup> Ca(II) was added to the sample and running buffer. <sup>d</sup> The elution volume corresponds to the maximum peak absorbance at 280 nm. <sup>e</sup> Molecular weights were estimated by the elution volumes of protein standards at pH 5.0–7.0.



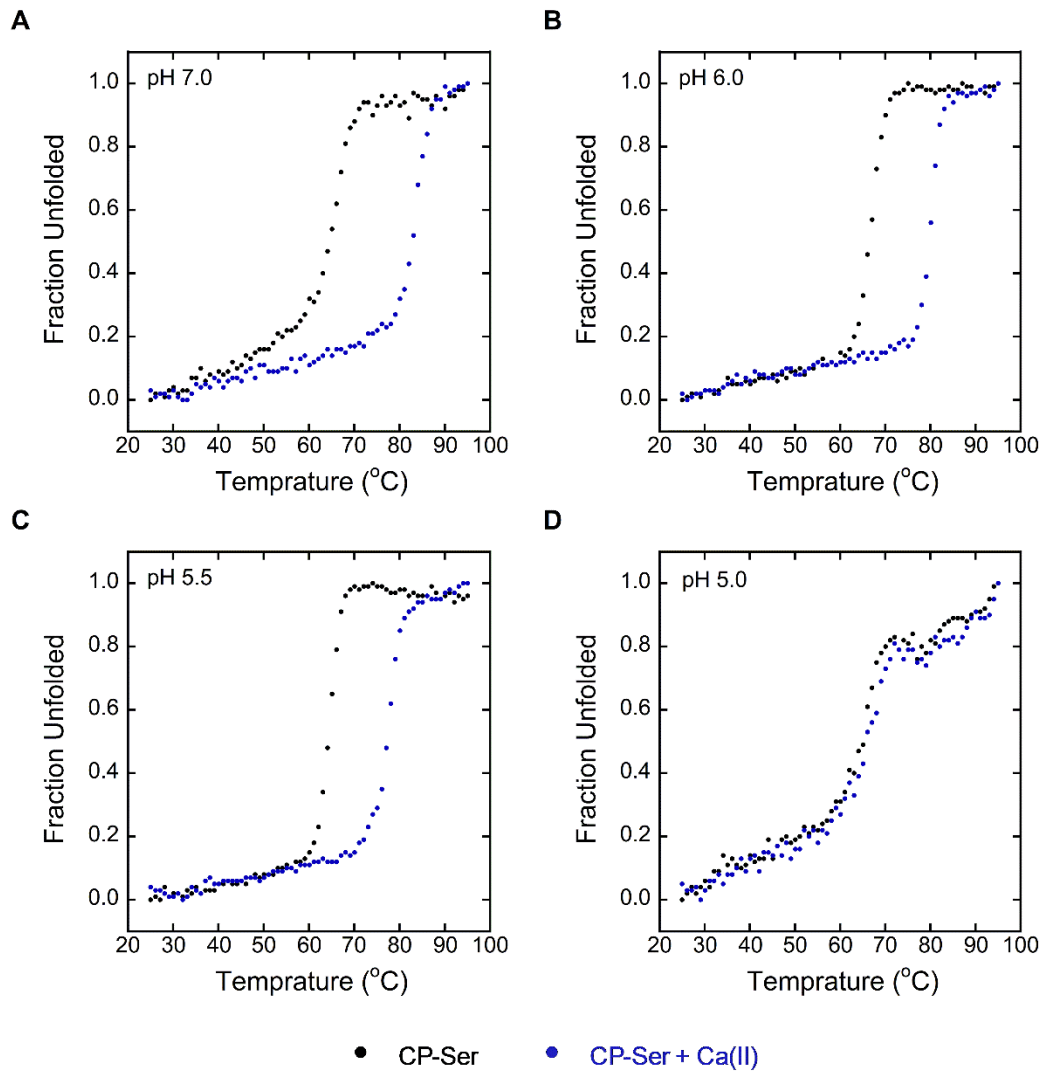
## Supporting Figures



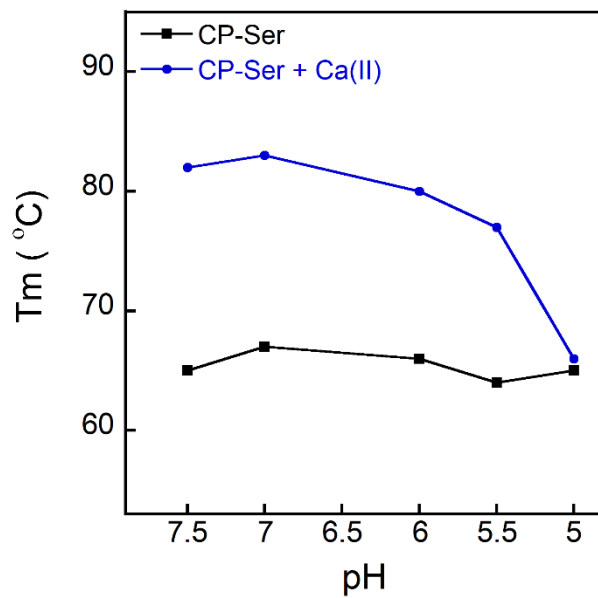
**Figure S1.** CD spectra of CP-Ser (10  $\mu$ M) in the absence (black) and presence (red) of 2 mM Ca(II) at pH 7.5 (A, 1 mM Tris-HCl), 7.0 (B), 6.0 (C), 5.5 (D) and 5.0 (E) (in 10 mM MES).



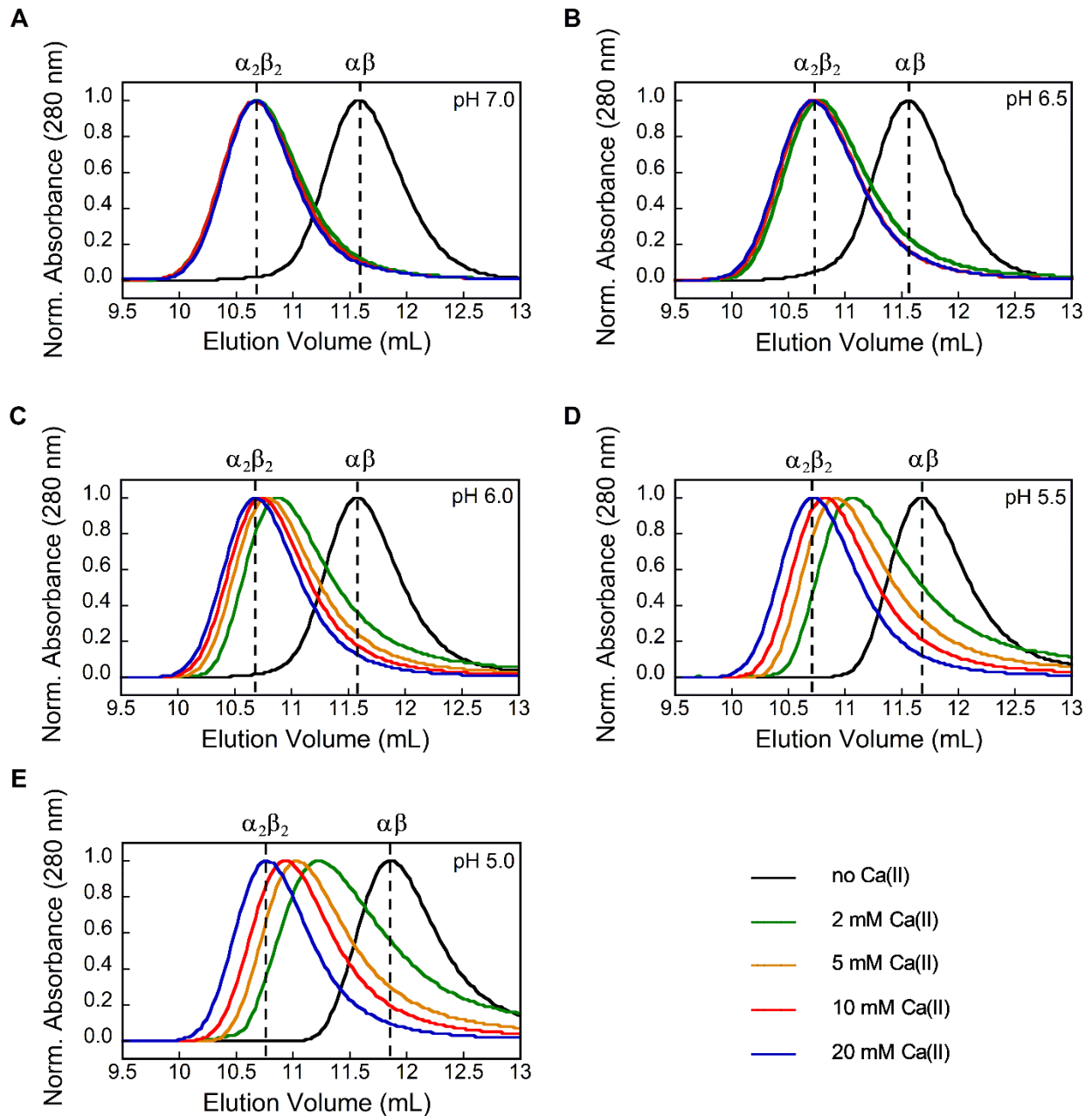
**Figure S2.** CD spectra of CP (10  $\mu$ M) in the absence (black) and presence (red) of 2 mM Ca(II) (10 mM MES) at pH 7.0 (A) and 5.0 (B).



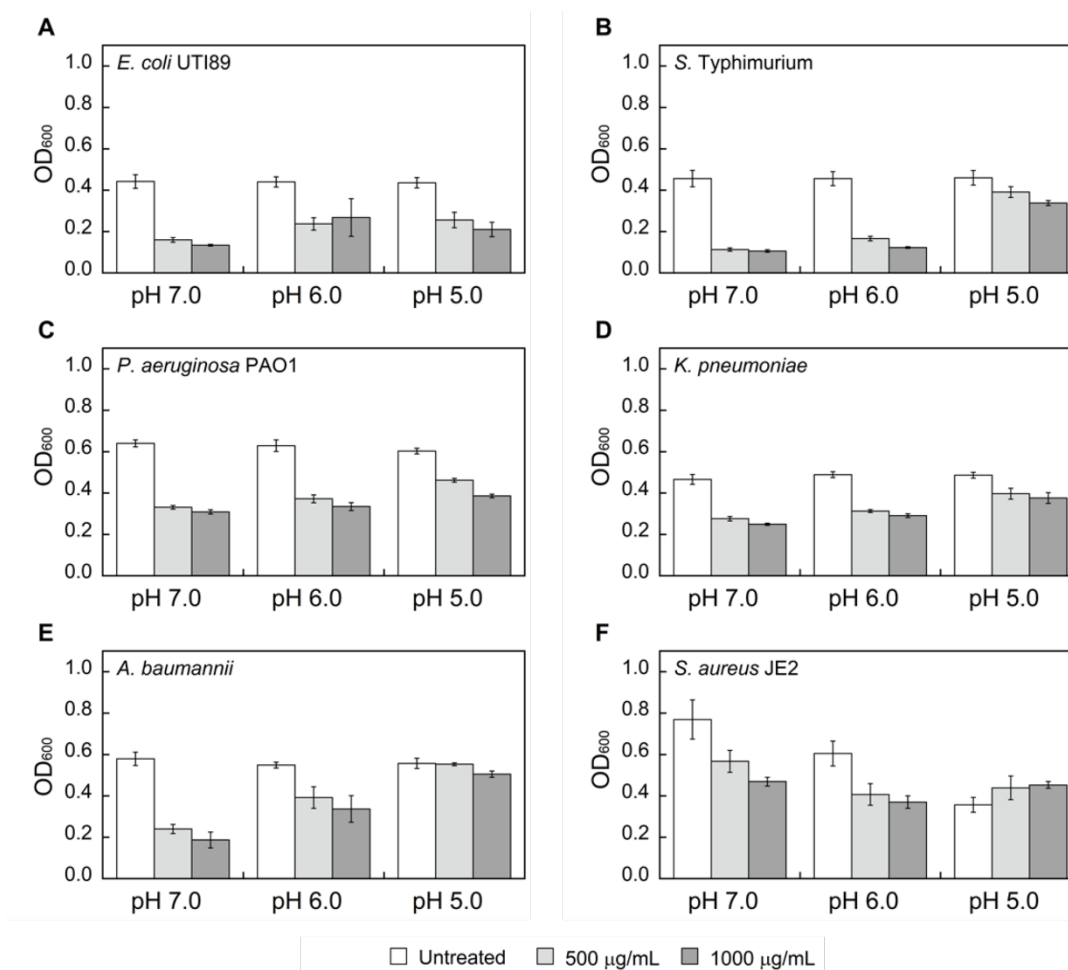
**Figure S3.** Representative thermal denaturation data for CP-Ser (10 μM) in the absence (black) and presence (blue) of 2 mM Ca(II) (10 mM MES) at pH 7.0 (A), 6.0 (B), 5.5 (C) and 5.0 (D). Each trace was normalized to the CD signal at 222 nm at 95 °C to obtain the fraction of unfolded protein.



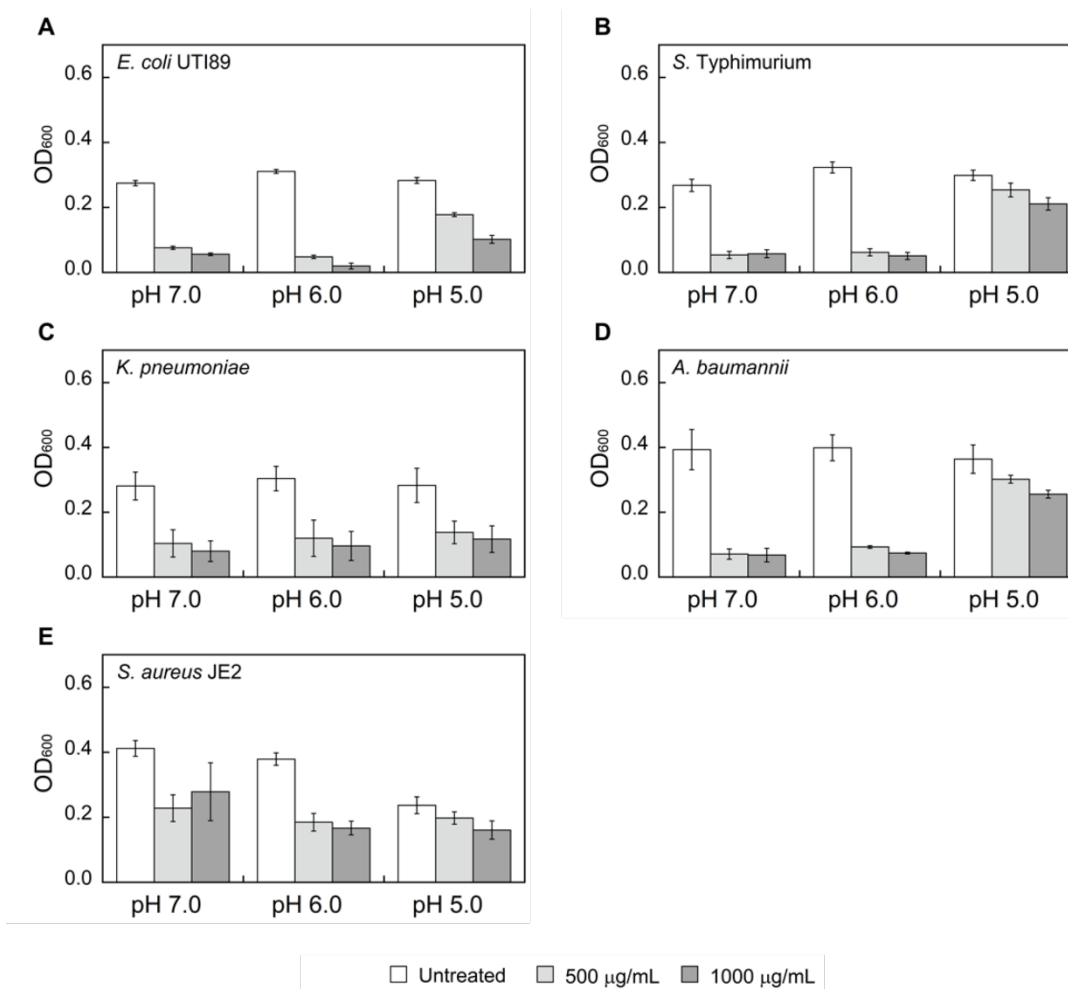
**Figure S4.** Melting temperature ( $T_m$ ) of CP-Ser as a function of pH in the absence (black) and presence (blue) of 2 mM Ca(II) (10 mM MES for pH 5.0–7.0, 1 mM Tris-HCl for pH 7.5).



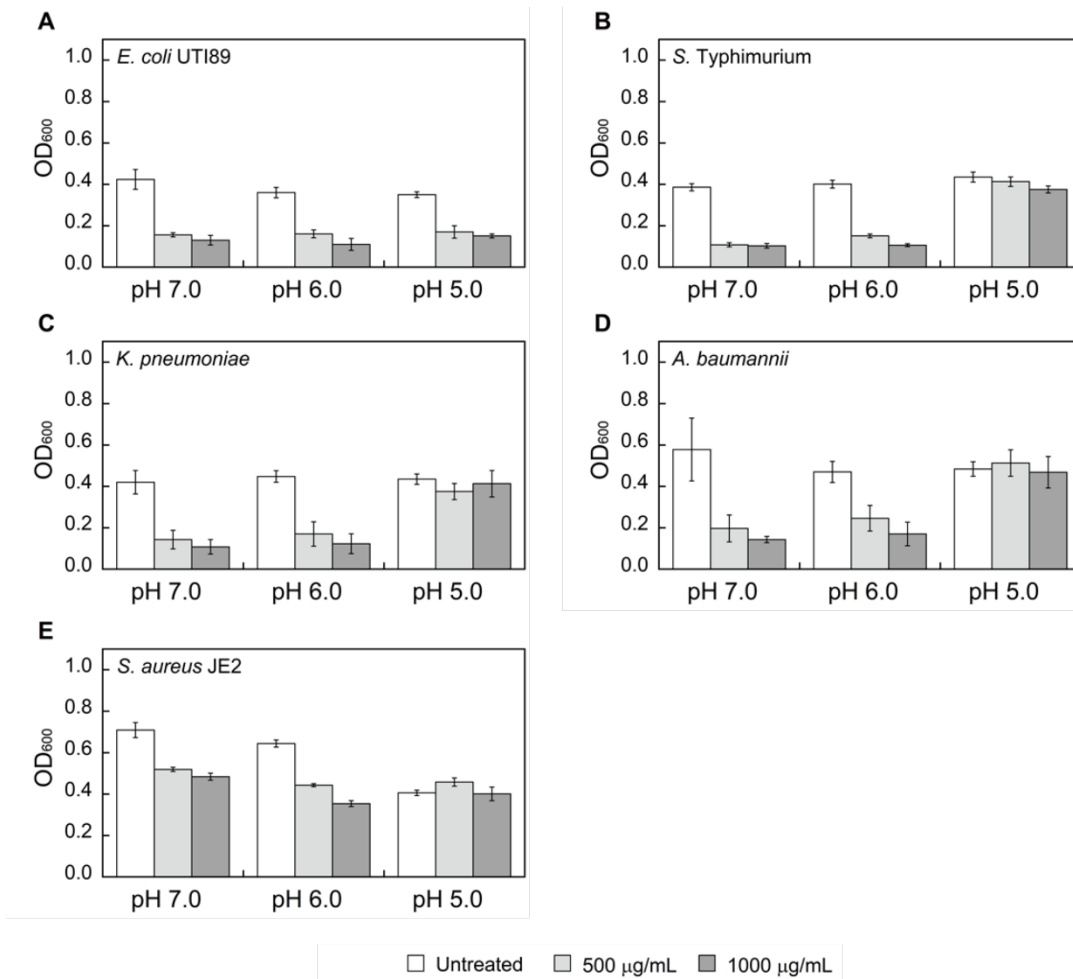
**Figure S5.** Analytical SEC chromatograms of CP-Ser (100 μM) in the absence and presence of 2, 5, 10 and 20 mM Ca(II) (75 mM MES, 100 mM NaCl, pH 5–7) at 4 °C. Each chromatogram was normalized to a maximum absorbance of 1. The vertical dashed lines indicate the peak elution volumes of the heterodimer ( $\alpha\beta$ ) and the heterotetramer ( $\alpha_2\beta_2$ ) forms of CP-Ser. Elution volumes and corresponding molecular weights are given in Table S4.



**Figure S6.** Growth inhibitory activity of CP-Ser against *E. coli* UTI89 (A), *S. Typhimurium* (B), *P. aeruginosa* PAO1 (C), *K. pneumoniae* (D), *A. baumannii* (E) and *S. aureus* JE2 (F) after 20 h incubation. Bacteria were grown in TSB:MES medium supplemented with 2 mM Ca(II) at 37 °C (mean  $\pm$  SDM, n  $\geq$  3). The indicated pH values correspond to the initial pH of the growth media. The pH of the cultures after 8 and 20 h incubation is provided in Table S3.

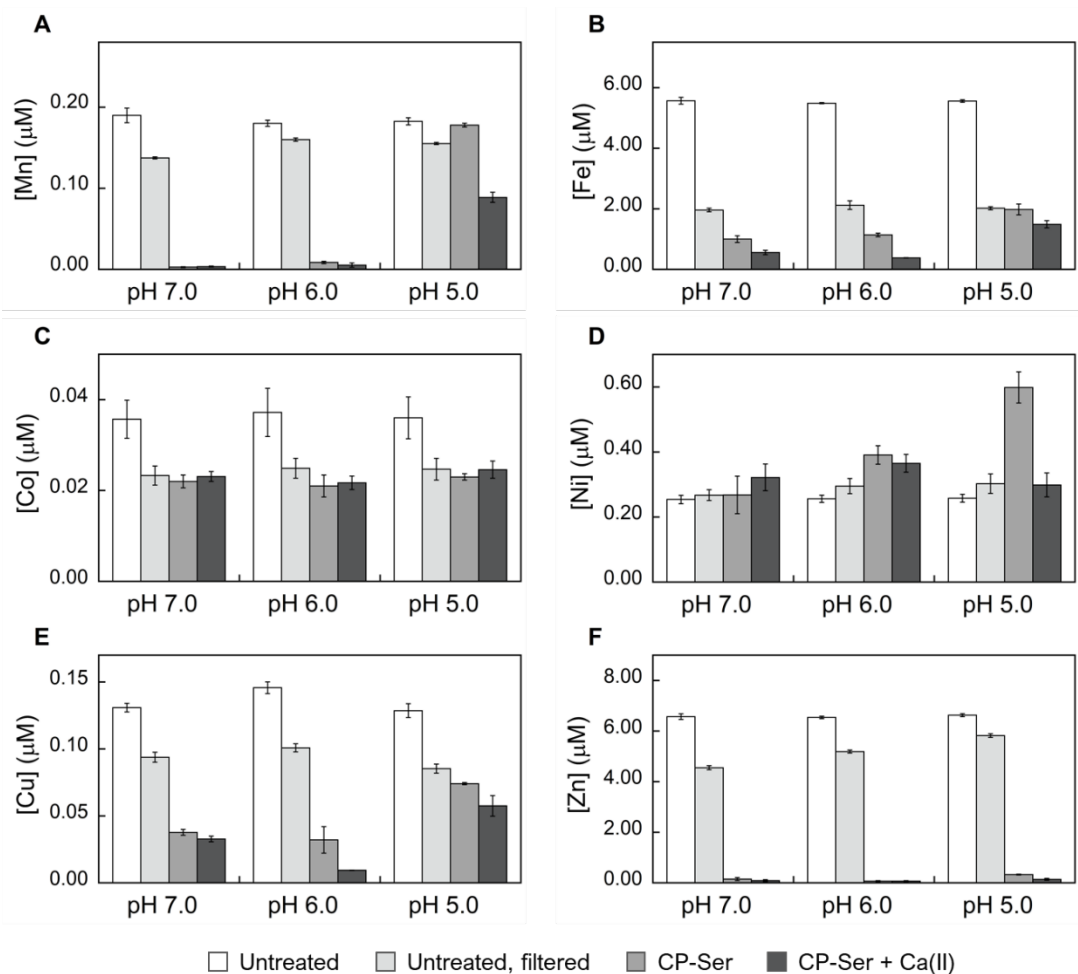


**Figure S7.** Growth inhibitory activity of CP against *E. coli* UT189 (A), *S. Typhimurium* (B), *K. pneumoniae* (C), *A. baumannii* (D) and *S. aureus* JE2 (E) after 8 h incubation. Bacteria were grown in TSB:MES supplemented with 2 mM Ca(II) at 37 °C (mean ± SDM, n ≥ 3). The indicated pH values correspond to the initial pH of the growth media. The pH of the cultures after 8 and 20 h incubation is provided in Table S3.

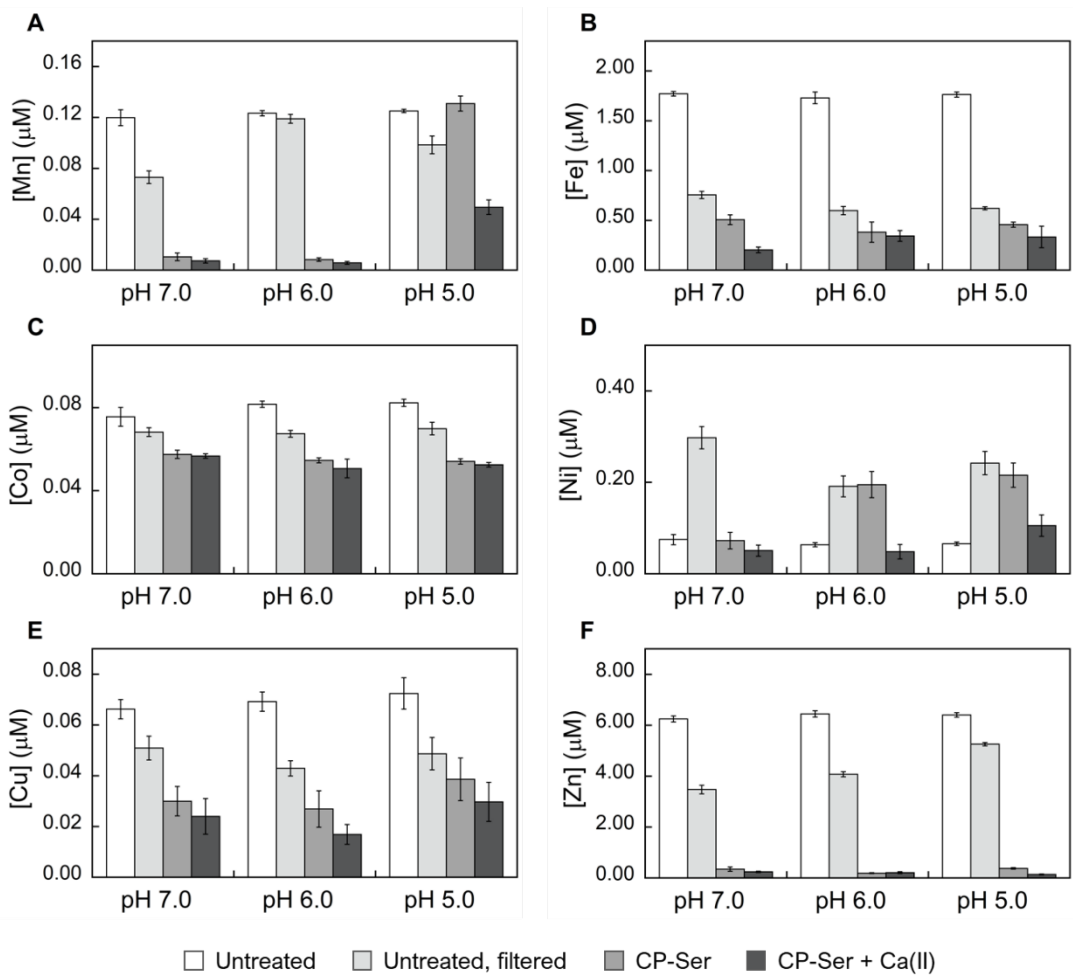


**Figure S8.** Growth inhibitory activity of CP against *E. coli* UT189 (A), *S. Typhimurium* (B), *K. pneumoniae* (C), *A. baumannii* (D) and *S. aureus* JE2 (E) after 20 h incubation. Bacteria were grown in TSB:MES supplemented with 2 mM Ca(II) at 37 °C (mean ± SDM, n ≥ 3). The indicated pH values correspond to the initial pH of the growth media. The pH of the cultures after 8 and 20 h incubation is provided in Table S3.

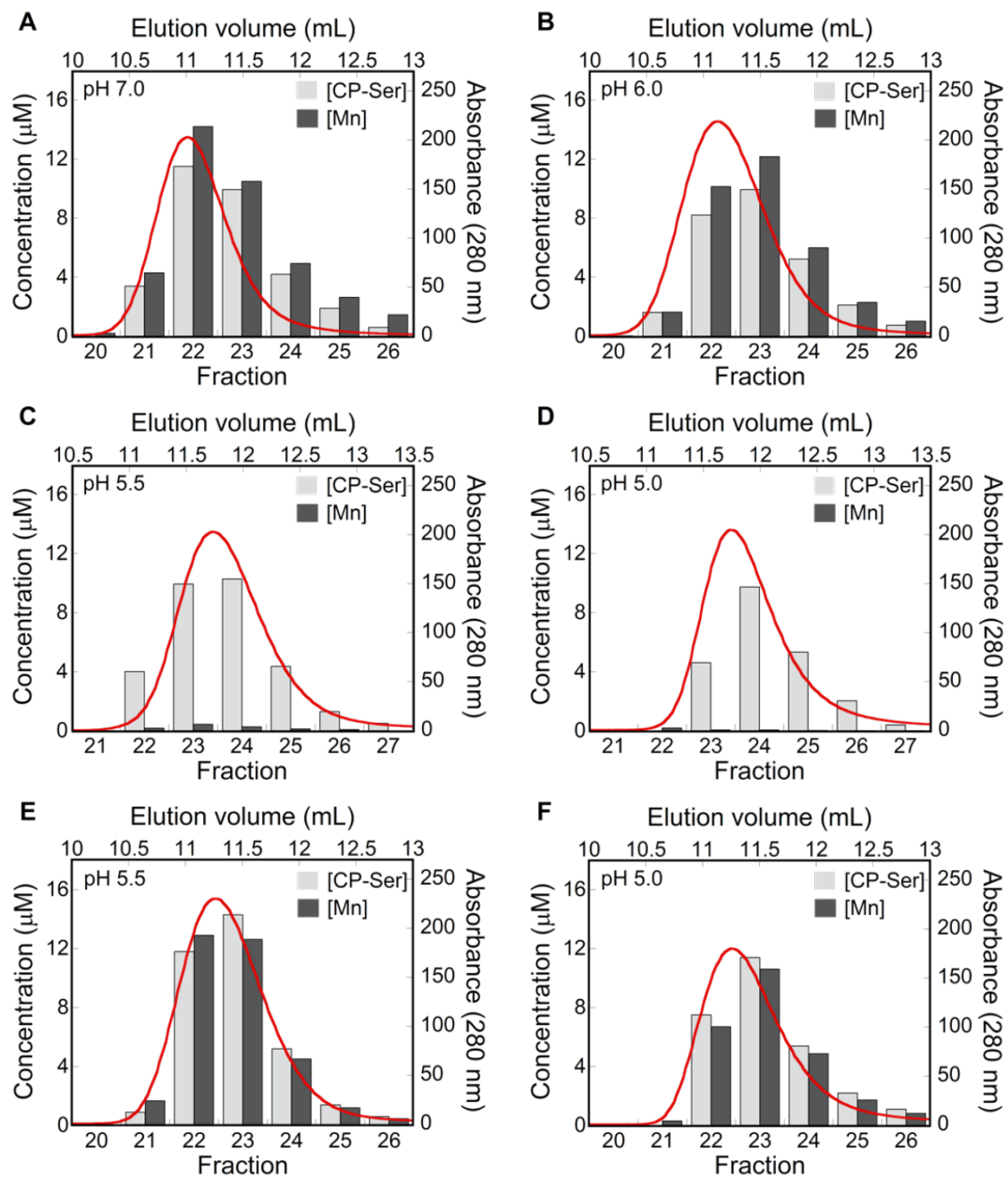




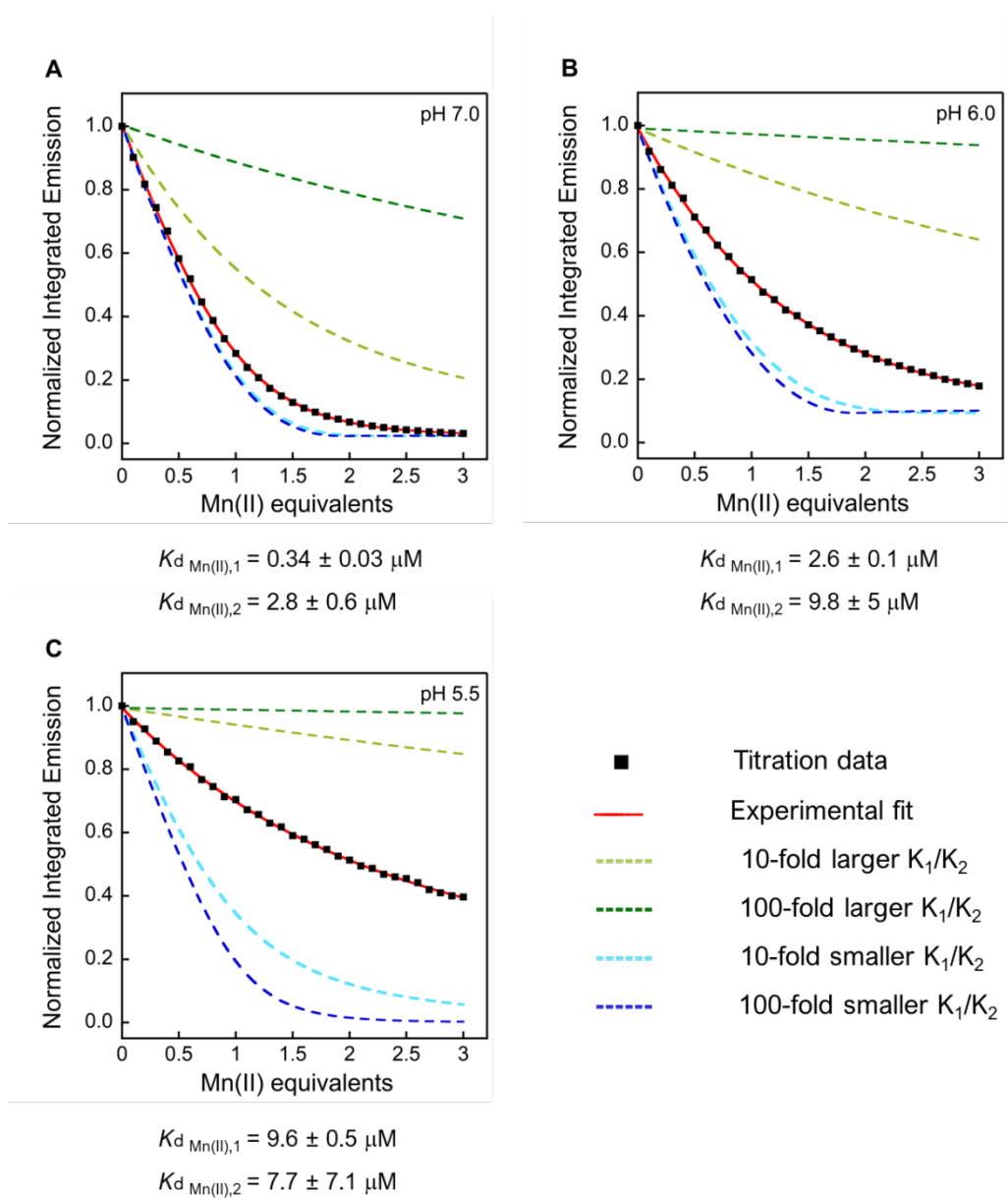
**Figure S9.** Metal analysis of MES:TSB medium treated with 250  $\mu\text{g}/\text{mL}$  of CP-Ser (10.4  $\mu\text{M}$ ) in the absence (medium gray bars) and presence (dark gray bars) of 2 mM Ca(II). After 20 h incubation (30  $^{\circ}\text{C}$ , 150 rpm), CP-Ser was removed by spin filtration, and the Mn (A), Fe (B), Co (C), Ni (D), Cu (E) and Zn (F) content of the filtrate was measured by ICP-MS. The metal content of untreated medium before (white bars) and after (light gray bars) spin filtration is presented for comparison. All data are averages  $\pm$  SEM,  $n \geq 4$ . Raw ICP-MS data are provided in Table S5.



**Figure S10.** Metal analysis of MES:LB medium treated with 250  $\mu\text{g/mL}$  of CP-Ser (10.4  $\mu\text{M}$ ) in the absence (medium gray bars) and presence (dark gray bars) of 2 mM Ca(II). After 20 h incubation (30  $^{\circ}\text{C}$ , 150 rpm), CP-Ser was removed by spin filtration, and the Mn (A), Fe (B), Co (C), Ni (D), Cu (E) and Zn (F) content of the filtrate was measured by ICP-MS. The metal content of untreated medium before (white bars) and after (light gray bars) spin filtration is presented for comparison. All data are averages  $\pm$  SEM,  $n \geq 4$ . Raw ICP-MS data are provided in Table S6.

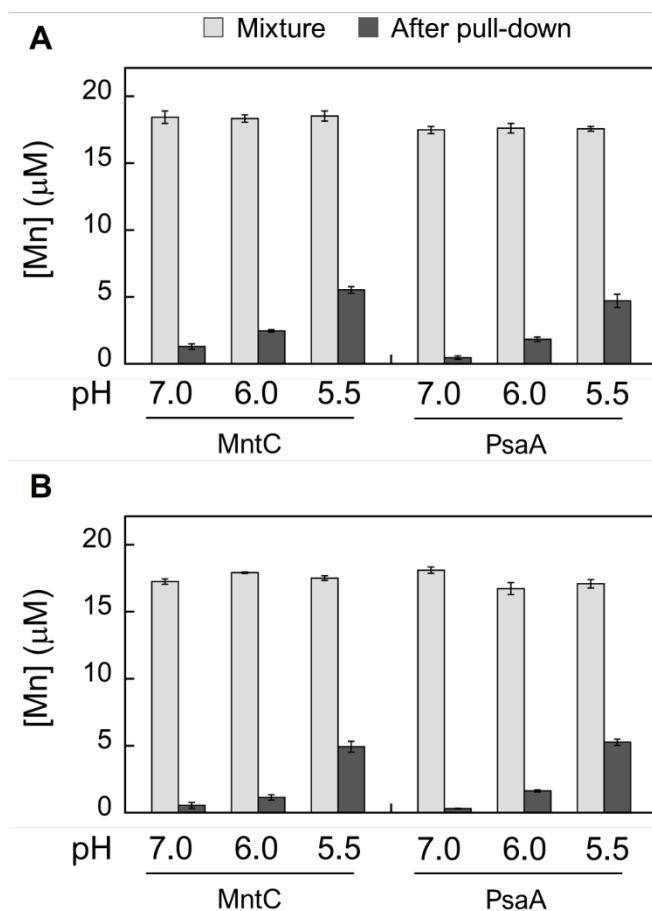


**Figure S11.** Mn retention by CP-Ser (200  $\mu\text{M}$ ) following SEC (75 mM MES, 100 mM NaCl, pH 5.0–7.0, 4  $^{\circ}\text{C}$ ) in the absence (A–D) or presence (E–F) of 4 mM Ca(II). Each plot contains a representative SEC chromatogram (red trace) and the quantification of protein and Mn concentrations in the collected fractions (bars).

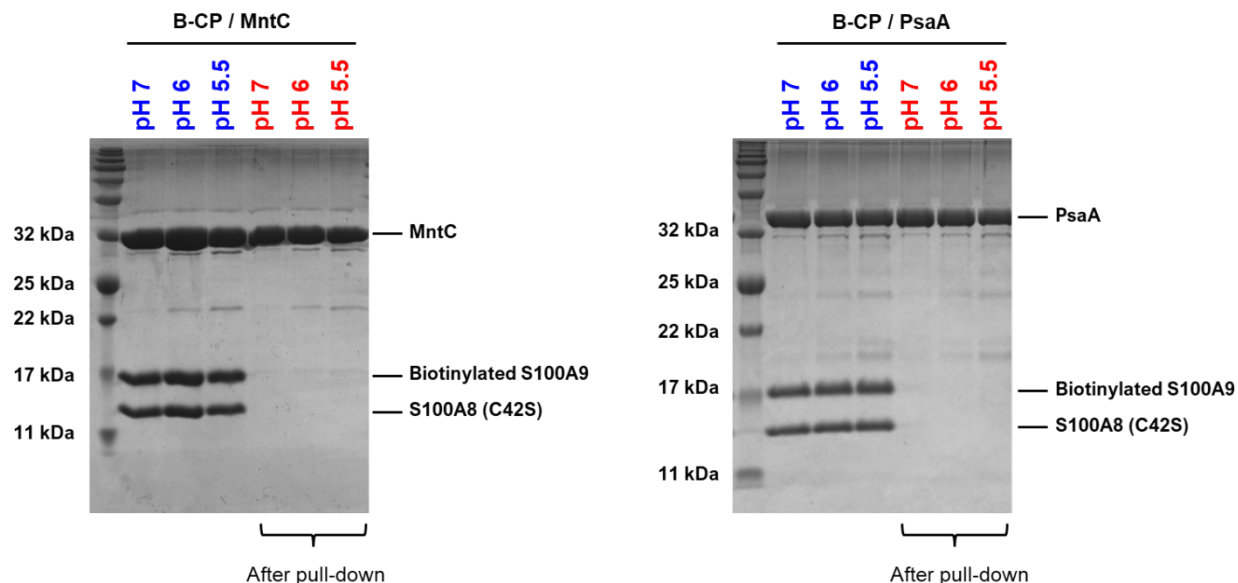


**Figure S12.** Titration of ZP1 (5  $\mu\text{M}$ ) with Mn(II) in MES buffer (75 mM MES, 100 mM NaCl, pH 5.5–7.0). A two-site binding model (see Experimental) was used to fit the normalized integrated emission data (black squares) with DynaFit (red solid line). The dashed lines represent simulated fits with higher and lower  $K_d$  values. All data are averages  $\pm$  SEM,  $n = 3$ .

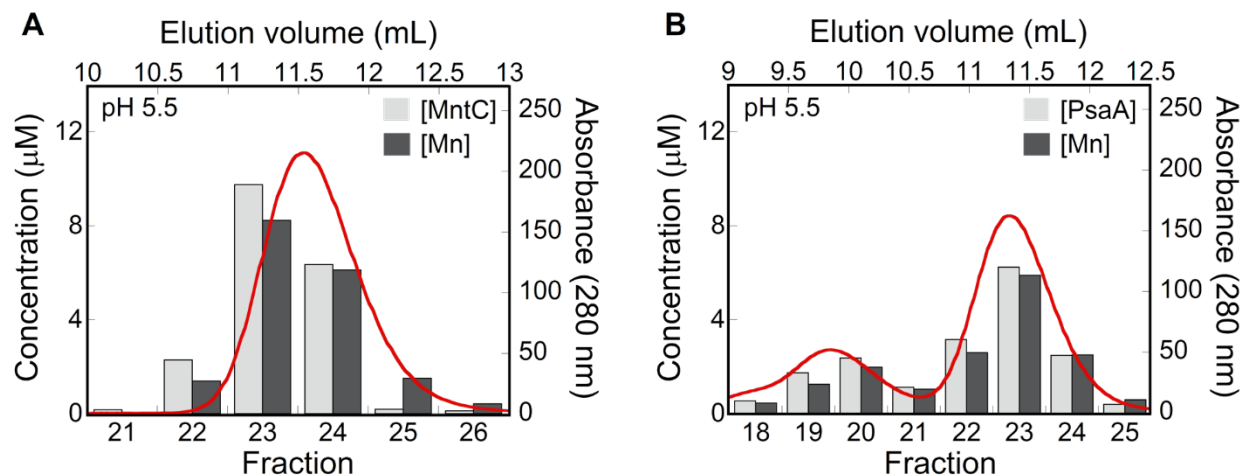
The ZP1 titration at pH 7.0 was performed for comparison with previously reported data, the latter of which afforded a  $K_{d,Mn(II)}$  value of 550 nM at pH 7.0.<sup>5</sup> This value and our value of 340 nM are in reasonable agreement.



**Figure 13.** ICP-MS analysis of Mn(II) in solution before (light gray bars) and after (dark gray bars) pull-down of mixtures containing either MntC or PsaA (20  $\mu$ M), B-CP (20  $\mu$ M) and Mn(II) (18  $\mu$ M) (75 mM MES, 100 mM NaCl, 1mM Ca(II), pH 5.5–7.0). (A) MntC/PsaA was preincubated with Mn(II) for 30 min followed by addition of B-CP. (B) B-CP was preincubated with Mn(II) for 30 min followed by addition of MntC/PsaA. All mixtures were incubated for 16 h at room temperature before pull-down. All data are averages  $\pm$  SEM, n = 3.



**Figure S14.** SDS-PAGE (15% Tris-glycine) analysis of B-CP pull-down assays. The gels correspond to the competition between either MntC (left) or PsaA (right) and B-CP for Mn(II). The protein ladder is P7712S from New England Biolabs.



**Figure 15.** Mn retention by MntC (left) and PsaA (right) (200  $\mu$ M) following SEC (75 mM MES, 100 mM NaCl, pH 5.5, 4  $^{\circ}$ C). Each plot contains a representative SEC chromatogram (red trace) and the quantification of protein and Mn concentrations in the collected fractions (bars). PsaA eluted as two species at 9.8 mL (73 KDa) and 11.3 mL (45 KDa), corresponding to a dimer and a monomer, respectively. Molecular weights were estimated by the elution volumes of protein standards at pH 5.5.

## Supplementary References

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