Supporting Information

Title: Valence-Driven Colorimetric Detection of Norovirus Protease via Peptide-AuNP Interaction

Authors: *Chuxuan Ling,*[a] *Zhicheng Jin,*[a] *Justin Yeung,*[b] *Elany Barbosa da Silva,*[c] *Yu-Ci Chang,*[d] *Tengyu He,*[d] *Wonjun Yim,*[d] *Anthony J. O'Donoghue,*[c] *Jesse V. Jokerst*[a,d,e*]

[a]Department of Nano and Chemical Engineering, University of California, San Diego, La Jolla, CA 92093, United States
[b]Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093, United States
[c]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, United States
[d]Program in Materials Science and Engineering, University of California, San Diego, La Jolla, CA 92093, United States
[e]Department of Radiology, University of California, San Diego, La Jolla, CA 92093, United States
***Corresponding author's email: jjokerst@ucsd.edu**

Materials and Methods

Materials

Gold(III) chloride hydrate ($HAuCl_4 \cdot 3H_2O$, ≥99.9%), Bis(p-sulfonatophenyl)phenylphosphine dihydrate dipotassium salt (BSPP, 97%), sodium citrate tribasic dihydrate (>99%), copper iodide(>99%), trifluoroacetic acid (TFA, HPLC grade, >99%), TFA (ReagentPlus®, 99%), piperidine (ReagentPlus®, 99%), DL-dithiothreitol (DTT, >99%), trypsin, thrombin (from human plasma), hemoglobin(Hgb), albumin from bovine serum (BSA) and automation compatible syringe filters (hydrophilic PTFE, 0.45 μm) were purchased from Sigma-Aldrich (St Louis MO). Sodium chloride (NaCl, certified ACS) and hydrochloric acid (certified ACS) were purchased from Fisher Chemical (Waltham, MA). Pyridine (ACS certified), N,N-diisopropylethylamine (DIPEA, >99%), thioanisole (>99%), 2,2′-(ethylenedioxy)diethanethiol (EDDET, 95%), sodium diphenylphosphinobenzene-3-sulfonate (DPPS, >90%), and triisopropylsilane (TIPS, >98%) were purchased from Tokyo Chemical Industry Co., Ltd (TCI). Sodium ascorbate was purchased from Spectrum Chemical (Gardena, CA). N,N–dimethylformamide (DMF), methylene chloride (DCM, certified ACS), dimethyl sulfoxide (DMSO, certified ACS), acetonitrile (ACN, HPLC grade), and ethyl ether (certified ACS were purchased from Fisher Scientific (Hampton, NH). Fmoc-Rink amide MBHA resin (0.67 mmol/g, 100-150 mesh), Fmoc-protected amino acids, and hexafluorophosphate benzotriazole tetramethyl uranium (HBTU) were purchased from AAPPTec, LLC (Louisville, KY). Fmoc-L-propargylglycine (Pra) was purchased from Combi Blocks Inc. (San Diego, CA). The azide-Cy5.5 was purchased from Lumiprobe Corp. (Hunt Valley, MD). Triphenylphosphine-3,3',3''-trisulfonic acid trisodium salt hydrate (TPPTS, 85%) was purchased from Beantown Chemical (Hudson, NH). The pooled whole human saliva, urine, and α-amylase were purchased from Lee Biosolutions, Inc. (Maryland Heights, MO). Disposable reaction vessel and the pressure caps for peptide-resin cleavage were purchased from Torviq Inc (Tucson, AZ). Ultrapure water (18 MΩ.cm) was obtained from a Milli-Q Academic water purification system (Millipore Corp., Billerica, MA). The TEM grids (formvar/carbon 300 mesh Cu) were purchased from Ted Pella (Redding, CA). The lumox 96-well cell culture plate (foil base) was from Sarstedt Inc. (Nümbrecht, Germany).

Methods

1. AuNPs synthesis and ligand exchange

Colloidal gold synthesis. The citrate-AuNPs (15 nm diameter, DLS) were prepared using the Turkevich method. An aqueous solution of sodium citrate tribasic dihydrate (150 mg, 5 mL) was rapidly injected into an aqueous solution of $HAuCl_4 \bullet 3H_2O$ (45 mg, 300 mL) under boiling conditions and vigorous stirring. The mixture was left to stir and boil for another 20 minutes, and then cooled to room temperature. The deep red dispersion was centrifuged at 18,000 g for 40 minutes for purification, and the supernatant was discarded. The resulting pellet of citrate-AuNPs was redispersed in DI water. DPPS, BSPP, TPPTS-AuNPs were then prepared by a ligand exchange method. The citrate-AuNP dispersion (50 mL, 3.4 nM) was vigorously stirred overnight with DPPS, BSPP, TPPTS (50 mg, 1 mL) in a round bottom flask, respectively. The mixtures were then filtered by syringe (hydrophilic PTFE, 0.45 mm), centrifuged, and then redispersed as the citrate-AuNPs procedure. The products were stored at 4˚C for future use.

2. Peptides preparation

2.1 Peptide synthesis and purification

Peptides were synthesized using an automated Eclipse™ peptide synthesizer (AAPPTec, Louisville, KY) through standard solid phase Fmoc synthesis on Rink-amide resin. Peptides were chain assembled by Fmoc-SPPS (solid-phase peptide synthesis) on rink-amide-MBHA-resin (0.67 mmol/g, 200 mg). Amino acid coupling was performed under the protection of nitrogen gas with 0.2 M Fmoc-amino acid (5 equiv.) in 3 mL DMF, 0.2 M HBTU in DMF (5 equiv.) in 3 mL DMF, 0.3 M DIPEA (7.5 equiv.) in 3 mL DMF, and 20% (v/v) piperidine in 2×4 mL DMF for each cycle. The number of coupling cycles were determined by the sequence analysis software tool (AAPPTec). The resulting peptides on the resin were transferred into a syringe filter (Torviq Inc,), and washed with three rounds of DCM (5 mL) each, followed drying under a vacuum. The peptides were then cleaved from the resin using a cleavage cocktail (5 mL) containing TFA (82.5%), EDDET (2.5%), phenol (5%), thioanisole (5%), and H_2O (5%). The mixture was incubated for 2 hours under room temperature with gentle shaking. After cleavage, the mixture was filtered, and the filtrate was precipitated and washed with three rounds of cold ether (25 mL, -20˚C), suspended in 50% ACN/H_2O (10 mL, v/v) to be lyophilized in a FreeZone Plus 2.5 freeze dry system (Labconco S5 Corp., Kansas, MO).

Peptide purification was carried out with a Shimadzu LC-40 HPLC system equipped with a LC-40D solvent delivery module, a photodiode array detector SPD-M40, and DGU-403 degassing unit. The crude sample was dissolved in an ACN/H_2O mixture (1:1, v/v) and injected with a volume of 2 mL on a Shim-pack GIS, C18 column (5 mm, 4.6×100 mm) and eluted at a flow rate of 3 mL/min over a 30 min linear gradient from 10% to 90% of ACN in water (with 0.05% TFA, HPLC grade). Fractions containing the pure peptide were confirmed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (positive ion mode) using the Bruker Autoflex Max MALDI-TOFMS instrument, and electrospray ionization mass spectrometry (ESI-MS) using the Micromass Quattro Ultima mass spectrometer in the Molecular MS Facility (MMSF) at UC San Diego. MALDI-TOF samples were prepared in an a-Cyano-4-hydroxycinnamic acid (HCCA)/H_2O mixture (1:1, v/v). ESI-MS samples were prepared in a MeOH/H_2O mixture (1:1, v/v).

Peptide concentrations were determined using a NanoDrop™ One UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA): The 31-method was applied for peptide quantification using an absorption coefficient of ε_{205} = 31 mL•mg^{-1} cm^{-1}.[1]

2.2 Proteolysis of peptides

A peptide solution (500 µL, 1 mg/mL) in Tris buffer (20 mM, pH 8.0, wih 150 mM NaCl and 5 mM DTT) was incubated with the SV3CP at a molar ratio of 100:1 (substrate: enzyme) at 37˚C for at least 48 hours to make a peptide fragment solution.

2.3 Peptide-dye synthesis

In a scintillation vial equipped with a stir bar, K1-pra peptide (1 µM, 1.1 mg), sodium ascorbate (2 equiv., 0.4 mg), and copper iodide (2 equiv., 0.38 mg) were dissolved in 800 µL DMSO/H_2O mixture (7:1, v/v). The azide-Cy5.5 (4 equiv., 2.8 mg) in 200 µL DMSO (anhydrate) was added into the reaction after 10 minutes of mixing under room temperature. Then, the reaction was moved to oil bath to react overnight under 60˚C, covered with an aluminum foil. All the steps were under

the protection of nitrogen. The crude mixture was then applied on a SPIRIT PROTEIN 300, C4 column (5 μm, 25×1 cm) from AAPPTec, LLC (Louisville, KY) and eluted at a flow rate of 3.0 mL/min over a 25 min linear gradient from 20% to 90% of ACN in water (with 0.05% TFA, HPLC grade). Preparative injections were monitored at 220 and 680 nm. Fractions containing the pure peptide-Cy5.5 were confirmed by MALDI-TOF MS, and then aliquoted (*i.e.*, $\varepsilon_{680} = 1.98 \times 10^5$ M^{-1} cm^{-1})and stored in dry/dark.

3. Norovirus SV3CP Protease Preparation

3.1 Norovirus SV3CP Protease Expression

SV3CP protease was expressed in *Escherichia coli* BL21(D3) cells from a pSV3C plasmid which was derived from pT7-7. A 20 mL starter E. coli culture in Luria-Bertani (LB) media with carbenicillin (50 μg/mL) was grown for 15 hours at 37˚C with shaking. 10 mL of starter culture was used to inoculate 1 L of LB/carbenicillin media and incubated at 180 rpm till the optical density reached 0.5. SV3CP expression was induced using 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at 37°C and 200 rpm. Cells were collected after 3 hours by centrifugation at 12,000 rpm for 20 min at 4°C. Then, the cell pellet was suspended in 50 mL of Buffer A consisting sodium phosphate buffer (10 mM, pH 7.65, 5 mM DTT). Approximately 2 mg of DNase I was added and cells were lysed by sonication on ice (2 s ON, 5 s OFF, 50% amplitude) for 5 cycles. The lysate was clarified by centrifugation at 12,000 rpm for 20 min at 4°C.

3.2 Norovirus SV3CP Protease Purification

Recombinant SV3CP was enriched from the supernatant using HiTrapCM FF (Cytiva) followed by HiTrapSP FF (Cytiva). Protein was clarified by passing through a 0.22-μm filter before loading on each column. All purification steps were carried out at 4°C. Protein was loaded in Buffer A and eluted in linear gradient of Buffer B that consisted of 10 mM Phosphate buffer pH 7.65, 5 mM DTT and 1 M NaCl. SDS-PAGE was employed to analyzed the purity of the fractions using a 4-12% Bis-Tris gel with MOPS running buffer and stained with Coomassie Blue. Fractions were also analyzed by a fluorescence-based activity assay using the peptide substrate, Ac-EFQLQ-7-amino-4-methylcoumarin from GenScript. Activity was measured in 384-well black plates on a Biotek Synergy HTX (excitiation 360 nm, emission 460 nm) at room temperature using 50 μM of fluorogenic substrate in Tris buffer (100 mM, pH 8.5 and 5 mM DTT). Fractions containing the highest enzyme activity were pooled and then buffer exchanged and concentrated using Buffer A in an Amicon Ultra centrifugal filter units with 3 kDa molecular weight cutoff (Millipore). The size-exclusion FPLC was the last step of purification with the Superose 6 Increase 10/300 GL (GE Healthcare) column using Buffer A with 100 mM NaCl added. Fractions were analyzed by SDS-PAGE and by a fluorescence-based activity assay. Fractions containing the most amount of active enzyme were pooled and stored at -80°C.

4 Operation window measurement

Parent/fragment peptides stock of the desired final concentration (*e.g.*, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 μM) in Tris buffer were added into the 96-well plate. Then, DPPS/BSPP/TPPTS-AuNPs (100 μL, 3.4 nM) were added to reach the final volume of 120 μL. Absorbance at 600 nm and 520 nm at room temperature were collected every minute for 1 hour. The ratiometric signal ($\lambda_{600/520}$) at 30 min was extracted for analyses. Each experiment has at least two replicates.

5 LoD measurement

K1 peptide and DPPS-AuNPs were used to detect SV3CP. The SV3CP enzymes of desired final concentration (1, 10, 100, 200, 350, 500, 750, 1000, 1500 nM) were mixed with the parent K1 peptide (c_{final} = 10 μM) in Tris buffer, and then incubated at 37˚C for 8 hours. Each experiment has at least two replicates. After 8 hours, the mixture was transferred to a 96-well plate and incubated with DPPS-AuNPs (100 μL, 3.4 nM). Absorbance at 600 nm and 520 nm at room temperature were collected every minute for 1 hour. The ratiometric signal ($\lambda_{600/520}$) at 30 min was extracted for analyses. The limit of detection was calculated following a reported statistical method:[2]

$$\mathrm{LoD_{int.}} = \mathrm{mean_{blank}} + 1.645 \times (\mathrm{SD_{blank}}) + 1.645 \times (\mathrm{SD_{low\ conc.\ sample}}) \quad \text{(S1)}$$

where 1.645 corresponds to a critical value from the standard normal distribution and is associated with a 95% confidence.

6 Probing Interparticle Interactions

An aggregation of DPPS-AuNPs was induced by thoroughly mixing a dispersion of DPPS-AuNPs (100 μL, 3.4 nM) and parent K1 peptide (10 μL, 285 μM) in a microtube. The mixture was then lightly centrifuged at 500 *g* for 1 minutes. The supernatant was discarded and the pellet was redispersed in a probing solution (100 μL) by sonication. These solutions include: DI water, HCl (10 mM), NaOH (10 mM), SDS (10 mM), DMSO (100%), DMF (100%), Triton X-100 (10 mM). The mixtures were then transferred to a 96-well plate and the absorbance at 600 nm and 520 nm at room temperature were collected every minute for 1 hour. The ratiometric signal ($\lambda_{600/520}$) at 30 min was extracted for analyses. The positive and negative controls were DPPS-AuNPs dispersion with K1 peptide and DPPS-AuNPs dispersion only, respectively.

7 Enzyme Kinetics

The kinetic model of an enzyme is mainly developed by Michaelis and Menten[3] and Henri,[4] with major improvement by Briggs-Haldane.[5] Simply, the enzyme and substrate reversibly form an enzyme-substrate complex, followed by the dissociation of the intermediate complex to produce the product with free enzyme, given as:

$$\mathrm{E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES \underset{k_{-cat}}{\overset{k_{cat}}{\rightleftharpoons}} E + P} \quad \text{(S2)}$$

where k_{on}, k_{off}, and k_{cat} are rate constants. The relationship between the initial velocity of product formation (υ) and the substrate concentration ($[S]_0$) is known as the classical Michaelis-Menten equation:

$$\upsilon = \frac{\upsilon_{max}}{K_M + [S]_0} \quad \text{(S3)}$$

where υ_{max} is the maximum velocity and K_M is the Michaelis-Menten constant. The subscript *0* denotes the total concentration. When $[S]_0 = K_M$, the initial velocity reaches ½ υ_{max}. Since the k_{cat} is the rate limiting step in enzymatic reactions, then:

$$v \equiv \frac{d[P]}{dt} = k_{cat}[ES] - k_{-cat}[E][P] \quad \text{(S4)}$$

Dissociation of the ES complex is usually considered as an irreversible process, due to the low affinity of enzyme to product, thus k_{-cat} is neglibile:

$$v \approx k_{cat}[ES] \; or \; V_{max} = k_{cat}[E]_0 \quad \text{(S5)}$$

Experimentally, in a 96-well plate the fluorogenic substrate was diluted in Tris buffer to reach a final $[S]_0$ of 5, 10, 20, 40, 80, and 160 μM (*e.g.*, with respect to a 30 μL final volume). SV3CP ($[E]_0$ = 2000 nM) was then added to each and the total volume was brought to 30 μL using TB buffer. The plate was then incubated at 37°C in a hybrid multi-mode microplate reader with 3 seconds of shaking before each cycle of reading and the photoluminescence intensity (excitation and emission at 630 and 720 nm respectively) was recorded over 12 hours with 2 minute intervals between each cycle. Measurements were done in triplicates. PL values at 30 minutes and 4 hours were averaged and recorded as PL_{30min} and PL_{max}, respectively. The PL_{max} was used to convert the PL_{30min} to initial velocity, V_0, which was then correlated to substrate concentration. Data was then fitted to the above Michaelis-Menten equation.

8 Matrix Test

The system's ability to detect SV3CP in different biological matrices was tested, including external breath condensate (EBC), saliva, urine, human plasma, 1% BSA, fecal matter, and Dulbecco's modified Eagle's medium (DMEM). SV3CP of desired amount (equivalent to 1000 nM in 120 μL) were spiked into the above matrices and incubated with parent K1 peptide at 37°C for 8 hours in microtubes. After 8 hours, the mixture was transferred to a 96-well plate and incubated with DPPS-AuNPs (100 μL, 3.4 nM). Absorbance at 600 nm and 520 nm at room temperature were collected every minute for 1 hour. The ratiometric signal ($\lambda_{600/520}$) at 30 min was extracted for analyses. Each experiment has at least two replicates.

9 Specificity Tests

9.1 Thermoregulated heat inactivation assay

SV3CP was incubated under 60˚C for 3 hours to be fully inactivated. Desired amount of SV3CP was then incubated with parent K1 in Tris buffer under 37˚C for 8 hours. After 8 hours, the mixture was transferred to a 96-well plate and incubated with DPPS-AuNPs (100 μL, 3.4 nM). Absorbance at 600 nm and 520 nm at room temperature were collected every minute for 1 hour. The ratiometric signal ($\lambda_{600/520}$) at 30 min was extracted for analyses. Positive and negative controls were DPPS-AuNPs and K1 peptide with active enzymes, and DPPS-AuNPs and K1 peptide only, respectively. Each experiment has at least two replicates.

9.2 Protein cross-test assay

The responsiveness of the sensor to SV3CP, bovine serum albumin (BSA), hemoglobin (Hb), salivary α-amylase (50 U/mL), thrombin (Tb), trypsin (Tp) was tested. These proteins of desirable amount ere spiked into Tris buffer to reach a final concentration of 1000 nM in 120 μL, followed incubation with parent K1 peptide (10 μM) under 37˚C for 8 hours. After 8 hours, the mixture was transferred to a 96-well plate and incubated with DPPS-AuNPs (100 μL, 3.4 nM). Absorbance at

600 nm and 520 nm at room temperature were collected every minute for 1 hour. The ratiometric signal ($\lambda_{600/520}$) at 30 min was extracted for analyses. Positive and negative controls were DPPS-AuNPs and K1 peptide with active enzymes, and DPPS-AuNPs and K1 peptide only, respectively. Each experiment has at least two replicates.

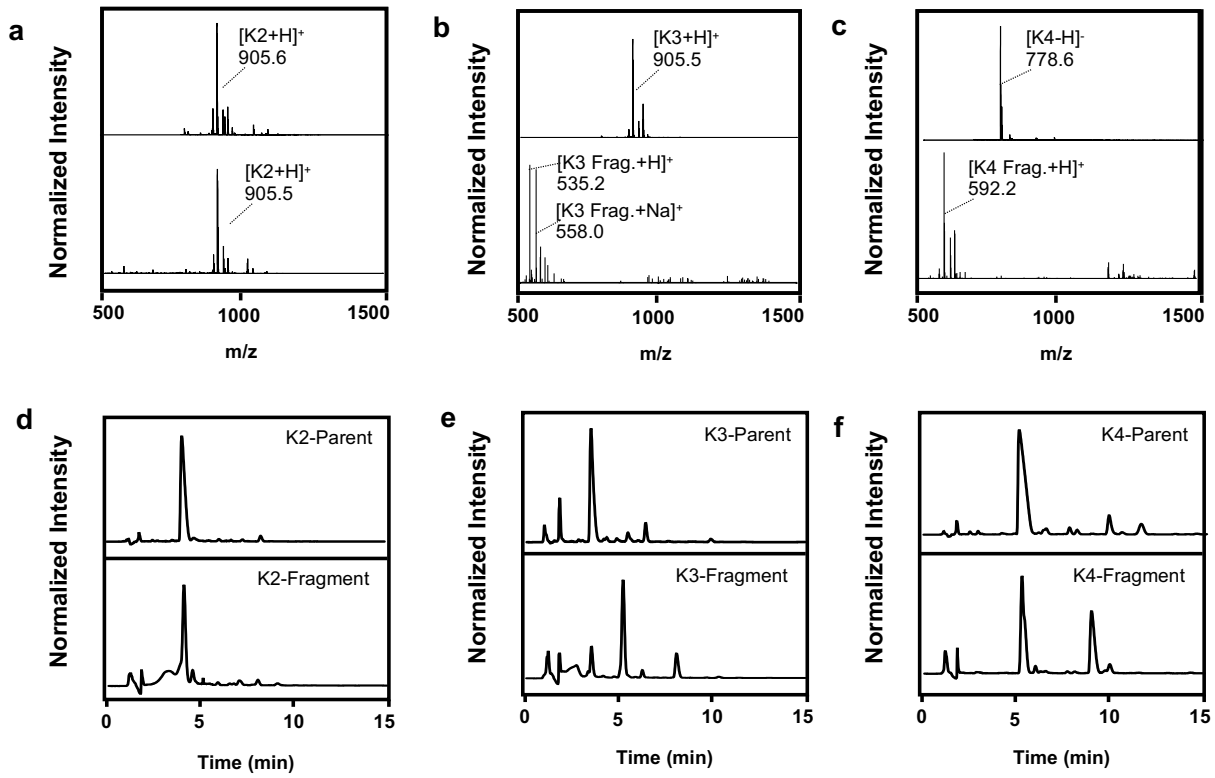

Figure S1. Confirmation of SV3CP-mediated cleavage of control peptides. MALD-TOF MS or ESI MS before (top) and after (bottom) incubation with SV3CP for peptides (a) K2, (b) K3, and (c) K4. HPLC before (top) and after (bottom) incubation with SV3CP for peptides (d) K2, (e) K3, and (f) K4. All peptides were incubated with SV3CP at a [E]:[S] ratio of 1:100 for 48 hours.

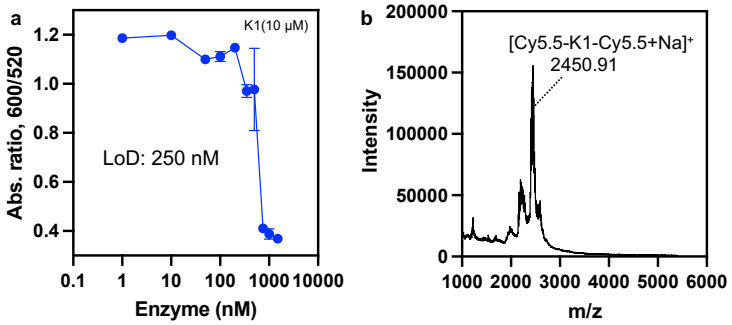

Figure S2. Limit of detection of SV3CP after 48 hours and MS data of peptide-fluorescent probe. (a) Ratiometric absorbance as a function of SV3CP concentration. The substrate was incubated with the protease in Tris buffer for 48 hours before addition of DPPS-AuNPs. (b) MALDI-TOF MS data of FRET probe (Cy5.5-K1-Cy5.5).

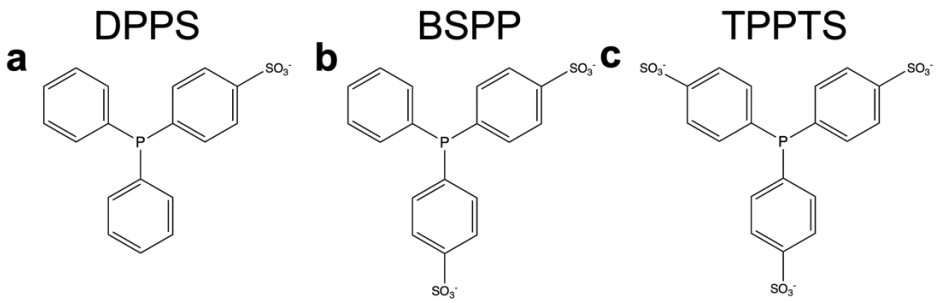

Figure S3. Chemical structures of ligands. (a) Diphenylphosphinobenzene sulfonate (DPPS). (b) Bis(p-sulfonatophenyl)phenylphosphine (BSPP). (c) Triphenylphosphine-3,3,3-trisulfonic acid (TPPTS).

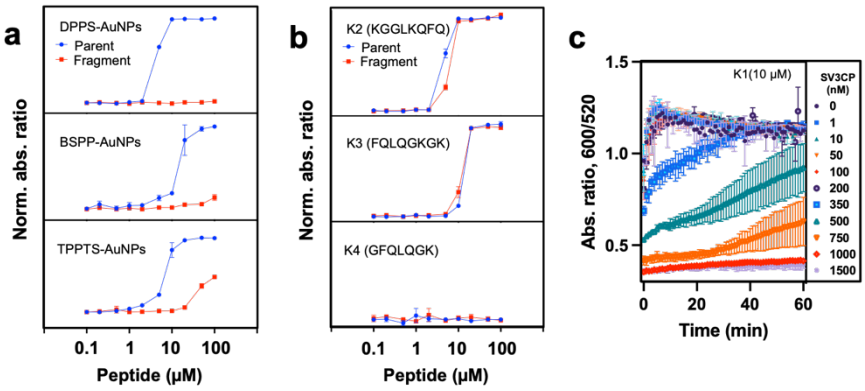

Figure S4. Working windows and kinetic. **(a)** Operation window of K1 based on the ratiometric signal from DPPS-AuNPs, BSPP-AuNPs, and TPPTS-AuNPs incubated with various amounts of K1 parent (blue) and fragments (red), respectively. **(b)** Operational windows based on K2, K3, and K4 incubated with DPPS-AuNPs, respectively. **(c)** Time progression of ratiometric absorbance in the enzyme assay where 10 μM K1 was incubated with increasing concentrations of SV3CP in Tris buffer (e.g., 1-1500 nM). Data points were read every 1 min for 1 h.

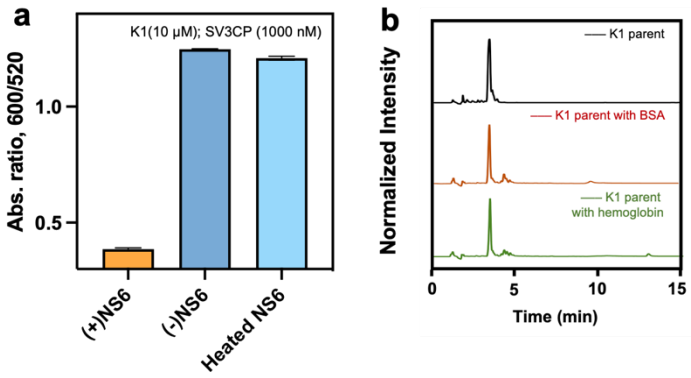

Figure S5. Heat denaturation and specificity. **(a)** Heat denaturation inactivation of SV3CP (1000 nM). SV3CP was heated at 60˚C for 3 hours before incubation with the substrate for 8 hours. **(b)** HPLC data of K1 parent, K1 parent with BSA, and K1 parent with hemoglobin, confirming intact K1 parent with the presence of these two proteins. This indicates that there is no cleavage happening with BSA or hemoglobin.

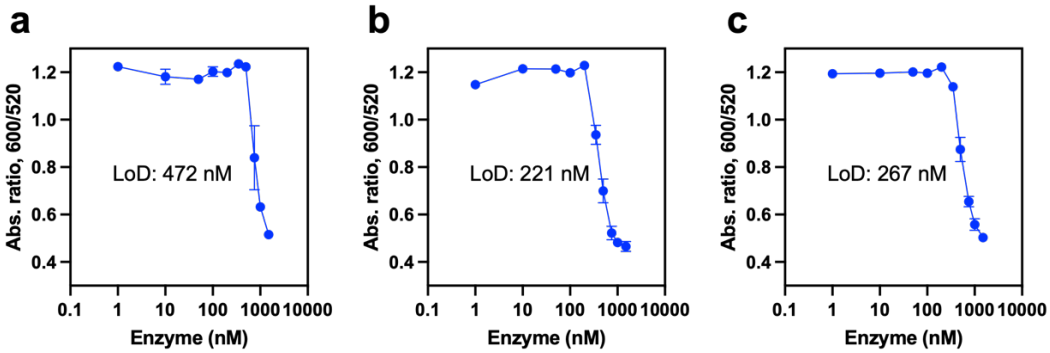

Figure S6. Limit of detection of SV3CP after 8 hours with EBC (a), urine (b), fecal matter (c). Ratiometric absorbance as a function of SV3CP concentration. The substrate was incubated with the protease in biological matrices for eight hours before addition of DPPS-AuNPs.

References:

(1) Anthis, N. J.; Clore, G. M. Sequence-specific determination of protein and peptide concentrations by absorbance at 205 nm. *Protein Science* **2013**, *22* (6), 851-858. DOI: https://doi.org/10.1002/pro.2253.

(2) Armbruster, D. A.; Pry, T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* **2008**, *29 Suppl 1* (Suppl 1), S49-52. From NLM.

(3) Johnson, K. A.; Goody, R. S. The Original Michaelis Constant: Translation of the 1913 Michaelis–Menten Paper. *Biochemistry* **2011**, *50* (39), 8264-8269. DOI: 10.1021/bi201284u.

(4) Henri, V. Recherches Physico-chimiques sur les Diastases. *Archivio di Fisiologia* **1904**, *1*, 299-324.

(5) Briggs, G. E.; Haldane, J. B. S. A Note on the Kinetics of Enzyme Action. *Biochemical Journal* **1925**, *19* (2), 338-339. DOI: 10.1042/bj0190338 (acccessed 7/11/2023).