Supporting Information

Rational Design of Antibody Protease Inhibitors

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Materials and Methods

Cloning of antibody Fab expression vector

The bovine antibody Fabs (bAb-TI and bAb-EI-L12) were created by replacing the "knob" domain (Cys108−Tyr146) in CDR3H region of BLV1H12 with the corresponding peptides (RC*TKSIPPIC*F for bAb-TI, and MC*TASIPPQC*Y for bAb-EI-L12, respectively, where C*C* represents a disulfide bond). For bAb-EI-L9, the peptide MC*TASIPPQC*Y was inserted in CDR3H region of BLV1H12 to replace sequence between Tyr105−Thr149. Gene fragments encoding the heavy chain of antibody Fabs were synthesized by IDT and amplified with the following primers:

5' gtcacgaattcgcaggtccagctgag

3' ccagctagctcactagtgatgatgatggtgatgtgtgtgagttttgtcgcaagatttgg

A C-terminal His_6-tag was attached to facilitate protein purification. The amplified gene fragments were digested with the restriction enzymes EcoRI-HF and NheI-HF (New England Biolabs, MA). The final mammalian expression vectors for the antibody Fabs were constructed by in-frame ligation of the assembled genes into the pFuse backbone vector (InvivoGen, CA) using T4 DNA ligase (New England Biolabs, MA) and confirmed by DNA sequencing.

For hAb-EI Fabs, the CDR3H sequences listed in Table 1 was inserted into the HuLys antibody heavy chain to replace the sequence between Ala96-Tyr105**.** Gene fragments encoding the heavy chain of these antibody Fabs with an N-terminal IL2 secretion signal peptide and C-terminal His_6-tag were amplified with the following primers:

5' gcactaagtcttgcacttgtcacgaattcg

3' ccagctagctcactagtgatgatgatggtgatgtgtgtgagttttgtc

The amplified genes were digested with the restriction enzymes EcoRI-HF and NheI-HF, and in-frame ligated into the pFuse backbone vector using T4 DNA ligase and confirmed by DNA sequencing.

Expression and Purification of Antibodies

The antibodies were expressed by transient transfection of FreeStyle 293F cells (Life Technologies, CA). 293F cells were maintained in shaker flasks (125 rpm) with freestyle 293 expression medium (Life Technologies) at 37 °C with 5% CO_2. Cells at a density of 10^6 cells/ml were transfected with heavy chain plasmid, light chain plasmid and 293fectin at a ratio of 2:1:6 following protocols suggested by Life Technologies. Expression medium containing secreted proteins was harvested every 48 h twice after transfection. Supernatant harvested at 48 h and 96 h were combined and subjected to affinity chromatography. Fabs fused with C-terminal His_6-tag were purified by Ni-NTA agarose (Qiagen, CA) according to the manufacturer's instruction. The eluted Fab proteins were buffer exchanged with PBS using desalting columns (Thermo Fisher Scientific, Rockford, IL). The Fabs were analyzed by SDS-PAGE gel in the presence and absence of dithiothreitol (DTT). The identities of the Fabs were further confirmed by electrospray mass spectrometry (ESI-MS) at Scripps Center for Metabolomics and Mass Spectrometry.

Peptide Synthesis

The control peptides aSFTI-1 with the sequence GRC*TKSIPPIC*FPD (where C*C* represents a disulfide bond) and HNEI with the sequence MC*TASIPPQC*Y were purchased from InnoPep, Inc (San Diego, CA). Peptides were dissolved in DMSO to make a 20 mg/mL stock solution.

Enzyme inhibition assays

Trypsin was purchased from Sigma-Aldrich (St. Louis, MO), catalog number T1426. Chymotrypsin was purchased from Sigma-Aldrich (St. Louis, MO), catalog number C3142. HNE and PR3 were provided by Elastin Products (Owensville, MO, USA), catalog number SE563 and ML734. Fixed concentrations of HNE (20 nM), bovine pancreatic trypsin (40 nM), or chymotrypsin (10 nM) were incubated with increasing concentrations of antibody Fabs (0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM and 1000 nM) in PBS buffer in opaque 96-well plates. After 15 min incubation at room

temperature, the corresponding fluorescent substrates MeOSuc-AAPV-AMC (Merck Millipore, final concentration 100 μM) for HNE, Bz-Arg-AMC (Sigma, final concentration 40 μM) for trypsin, or Suc-AAPF-AMC (Merck Millipore, final concentration 100 μM) for chymotrypsin were added to each well at the indicated final concentrations. Fluorescence was monitored on a Molecular Devices Spectramax fluorescence plate reader at 460 nm with excitation at 380 nm for 30 min. Residual enzyme activity was calculated using the slope of fluorescence increase in the first 10 min. For the PR3 inhibition assay, 15 nM PR3 was incubated with increasing concentrations of antibodies in PBS buffer. After 15 min incubation, 0.25 mM substrate Boc-Ala-Ala-Nva-SBzl (Elasin Co.) and 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma) in DMSO were added (with final DMSO concentration being 10%). Absorbance at 405 nm was recorded on a plate reader (Molecular Devices). Percentage of enzyme inhibition was plotted using GraphPad Prism 6 software (GraphPad Prism, La Jolla, CA, USA) and the K_i value was calculated using the Morrison tight-binding equation provided in the software. For control experiments, aSFTI-1 peptide, bAb-TI and bAb were used as controls for the trypsin inhibition assay; HNEI peptide and hAb-EI-L7-AA were used as controls for the HNE inhibition assay; PMSF was used as a control for the chymotrypsin inhibition assay to valid the experiment; His-tagged recombinant elafin was used as a positive control for the PR3 inhibition assay.

Biolayer interferometry

Biolayer interferometry experiments were performed using an Octet RED instrument (ForteBio, Inc.). Briefly, his-tag labeled Fabs at 1 μM in kinetics buffer (PBS, 0.01% BSA and 0.002% Tween 20) was immobilized onto a Ni-NTA coated biosensor. Each biosensor was then incubated with increasing concentrations of bovine trypsin (12.5 nM, 25 nM, 50 nM, 100 nM and 200 nM) or HNE (6.25 nM, 12.5 nM, 25 nM, 50 nM and 100 nM). The binding kinetics were monitored in real time with 4 min association and 4 min dissociation times. Kinetic parameters (k_{on}, k_{off}) and K_d were obtained by fitting the data to a 1:1 binding mode using Octet system software.

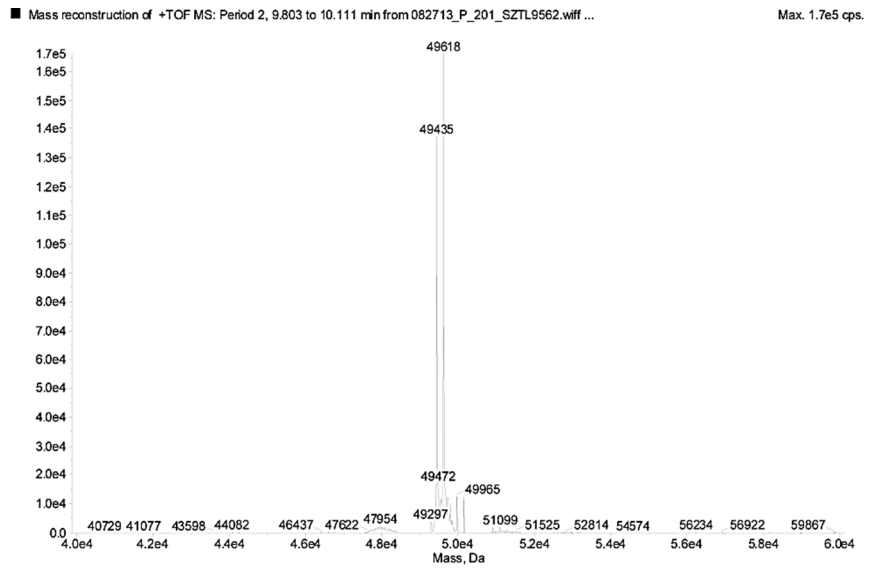

Figure S1. ESI-MS of the bAb-TI Fab. Exp: 49635 Da; Obs: 49618 Da and minor peak 49435 Da (matching the mass of antibody without Gln1 and Ala2).

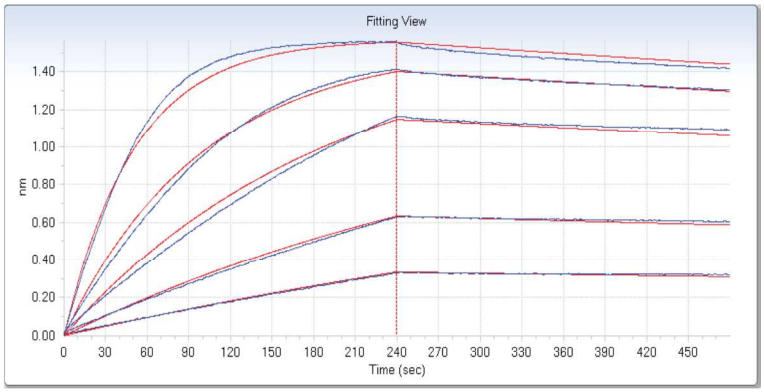

Figure S2. Kinetic characterization of bAb-EI and trypsin interaction by biolayer interferometry. Blue curves represent experimental data and red curves represent the curve fit. Briefly, his-tag labeled Fabs at 1 μM were immobilized onto a Ni-NTA coated biosensor. Each biosensor was incubated with a different concentration of bovine trypsin (12.5 nM, 25 nM, 50 nM, 100 nM and 200 nM). The binding kinetics were monitored in real time with 4 min association and 4 min dissociation times. Kinetic parameters (k_{on}, k_{off}) and K_d were obtained by fitting the data to a 1:1 binding mode using Octet system software.

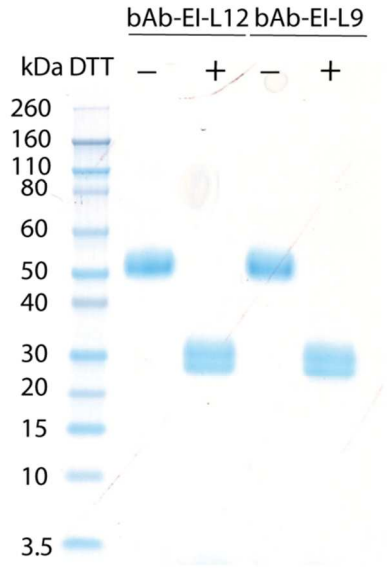

Figure S3. SDS-Page gel of purified bAb-EI Fabs in the absence (-) or presence (+) of DTT.

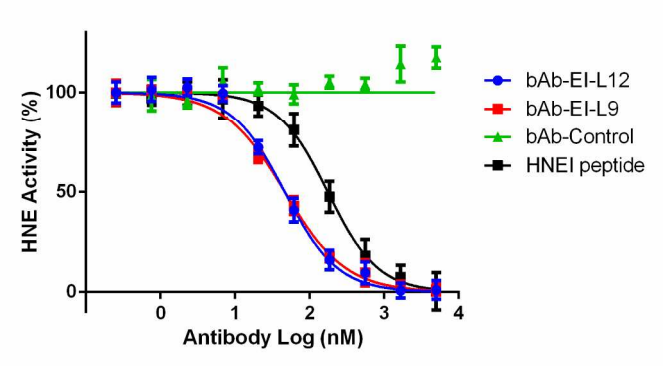

Figure S4. Engineered anti-HNE antibodies potently inhibit HNE enzymatic activity. HNE at 20 nM was incubated with increasing concentrations of bAb Fabs for 15 min, followed by the addition of 100 μM MeOSuc-AAPV-AMC. Residual enzyme activity was monitored using a plate reader (380 nm excitation and 460 nm emission). Assays were performed in triplicate.

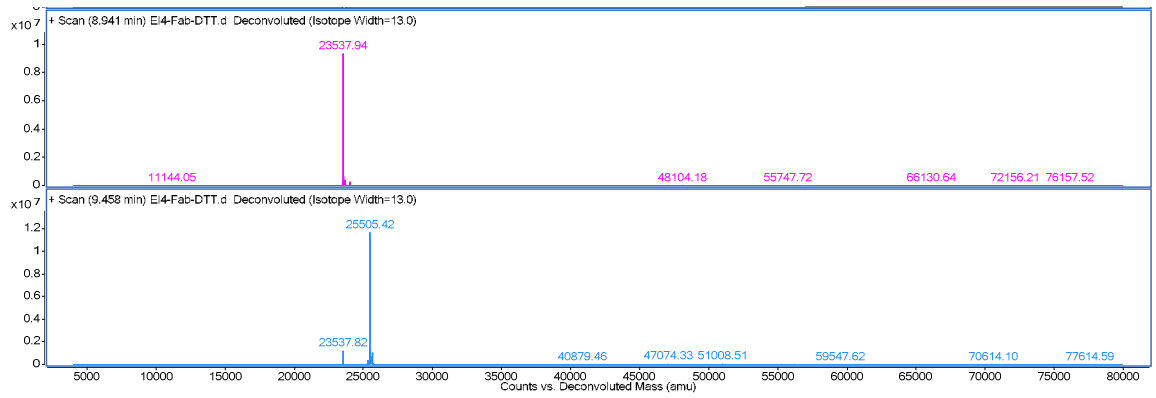

Figure S5. ESI-MS of hAb-EI-L4 Fab treated with DTT. Light chain (Exp: 23541 Da; Obs: 23538 Da); Heavy chain (Exp: 25525; Obs: 25505)

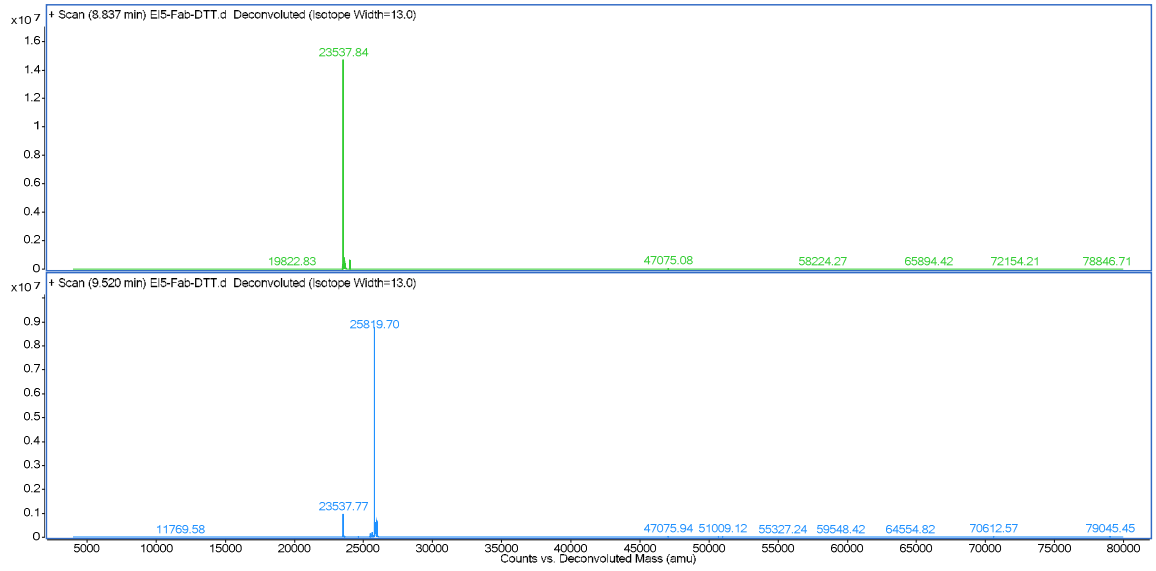

Figure S6. ESI-MS of hAb-EI-L5 Fab treated with DTT. Light chain (Exp: 23541 Da; Obs: 23538 Da); Heavy chain (Exp: 25840; Obs: 25820)

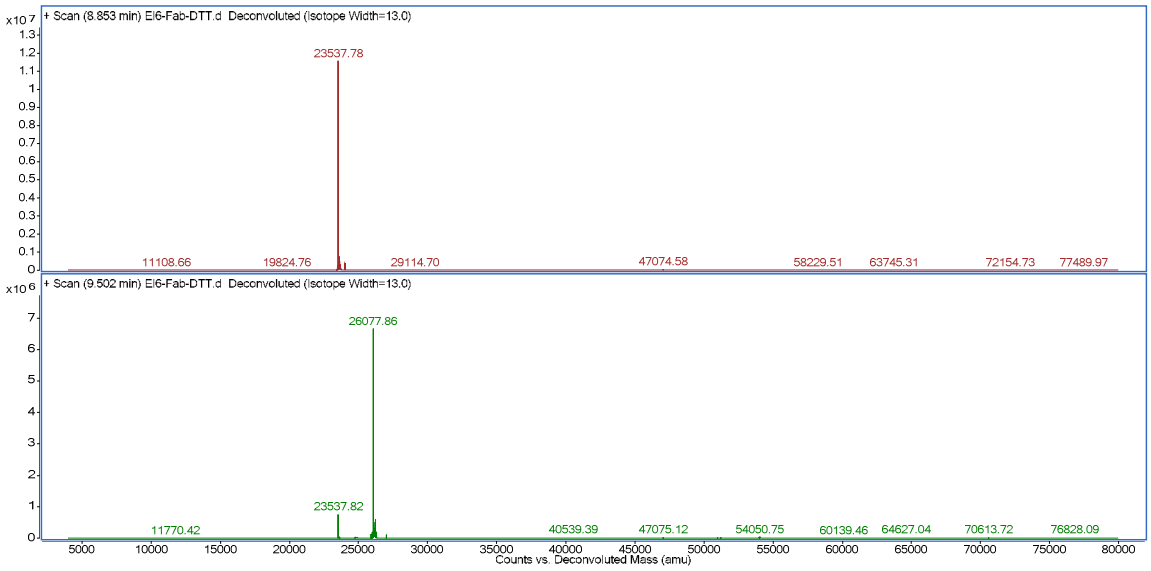

Figure S7. ESI-MS of hAb-EI-L6 Fab treated with DTT. Light chain (Exp: 23541 Da; Obs: 23538 Da); Heavy chain (Exp: 26098; Obs: 26078)

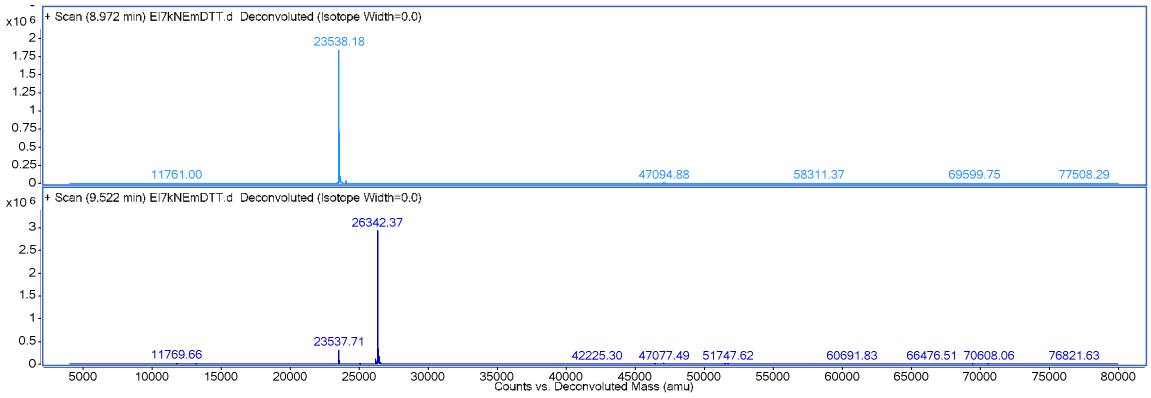

Figure S8. ESI-MS of hAb-EI-L7 Fab treated with DTT. Light chain (Exp: 23541 Da; Obs: 23538 Da); Heavy chain (Exp: 26362; Obs: 26342)

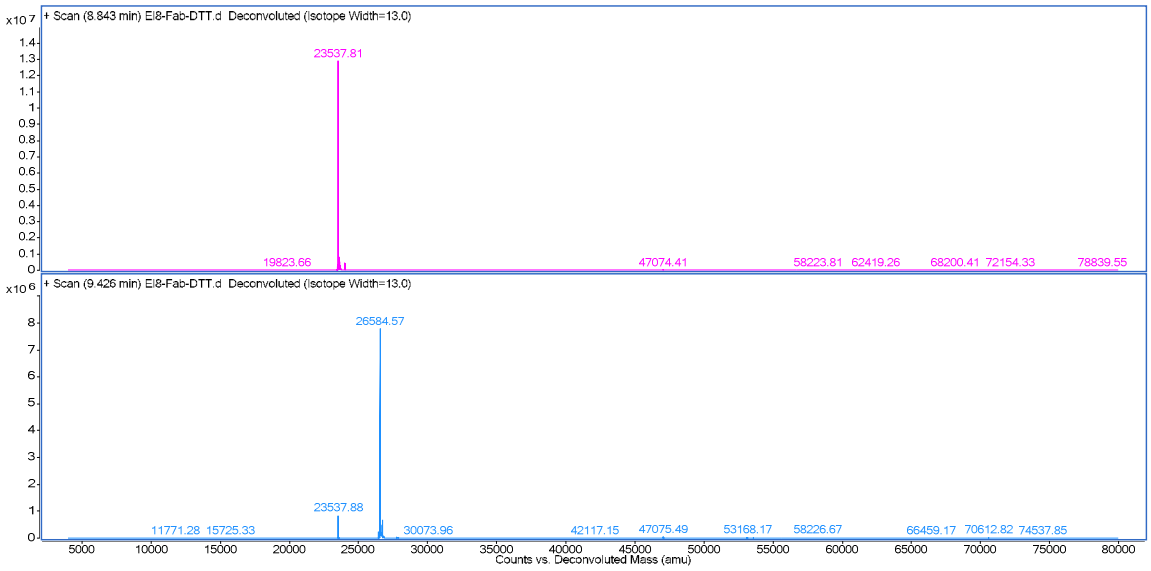

Figure S9. ESI-MS of hAb-EI-L8 Fab treated with DTT. Light chain (Exp: 23541 Da; Obs: 23538 Da); Heavy chain (Exp: 26604; Obs: 26584)

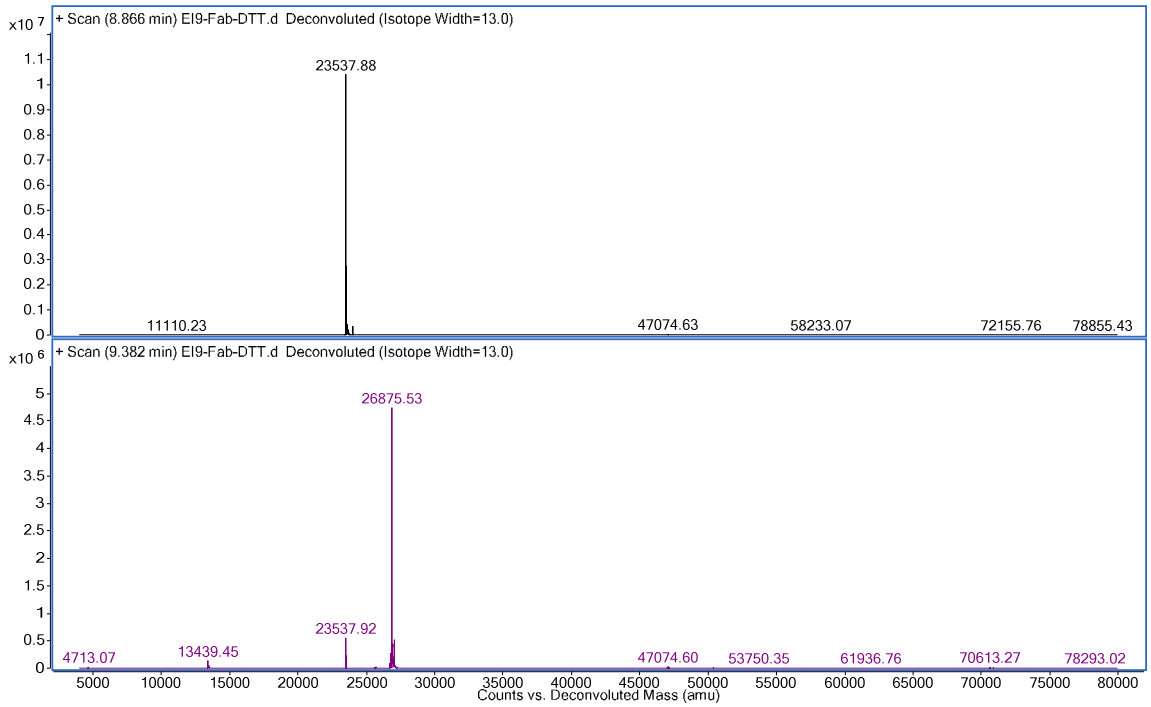

Figure S10. ESI-MS of hAb-EI-L9 Fab treated with DTT. Light chain (Exp: 23541 Da; Obs: 23538 Da); Heavy chain (Exp: 26896; Obs: 26875)

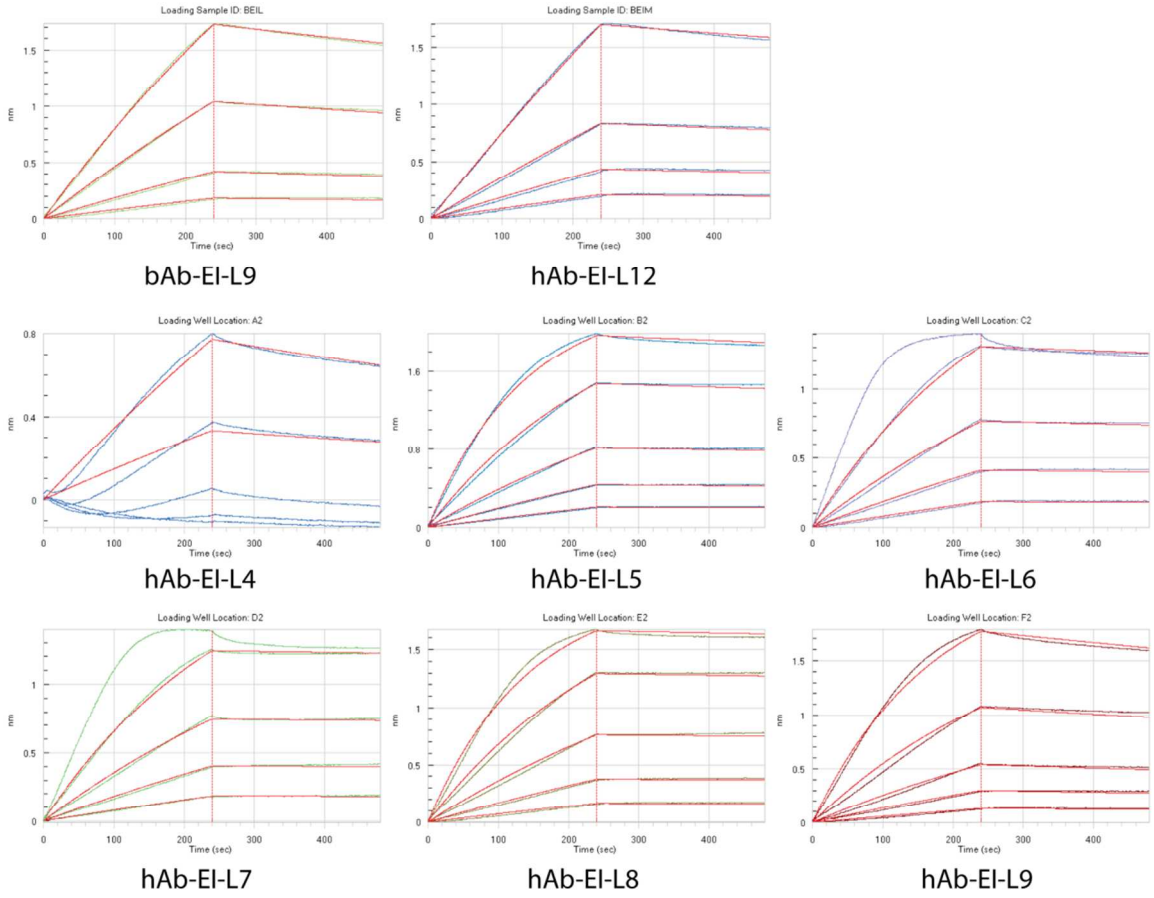

Figure S11. Kinetic characterization of engineered HNE inhibitory antibodies by biolayer interferometry. Colored curves represent experimental data and red curves represent the curve fit. Briefly, his-tag labeled Fabs at 1 μM were immobilized onto a Ni-NTA coated biosensor. Each biosensor was incubated with a different concentration of bovine trypsin (6.25 nM, 12.5 nM, 25 nM, 50 nM and 100 nM). The binding kinetics were monitored in real time with 4 min association and 4 min dissociation times. Kinetic parameters (k_{on}, k_{off}) and K_d were obtained by fitting the data to a 1:1 binding mode using Octet system software.

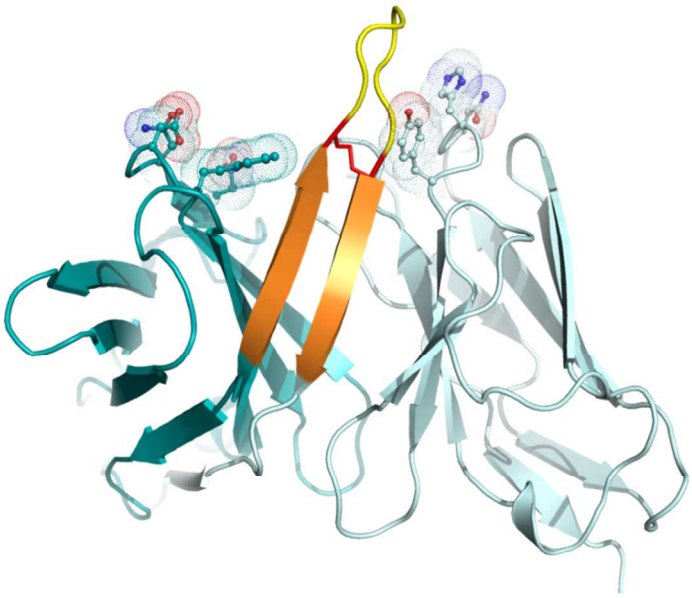

Figure S12. A model of the potential hAb-EI-L7 structure (LC in white and HC in cyan) based on the crystal structure of HuLys (PDB code 1BVK). The 7 residue-long anti-parallel β-strand of the CDR3H is shown in orange with the active disulfide-bridged (red) hairpin peptide displayed in yellow. Residues exposed on the potential interaction surface are marked with side chains shown in ball-and-stick model.

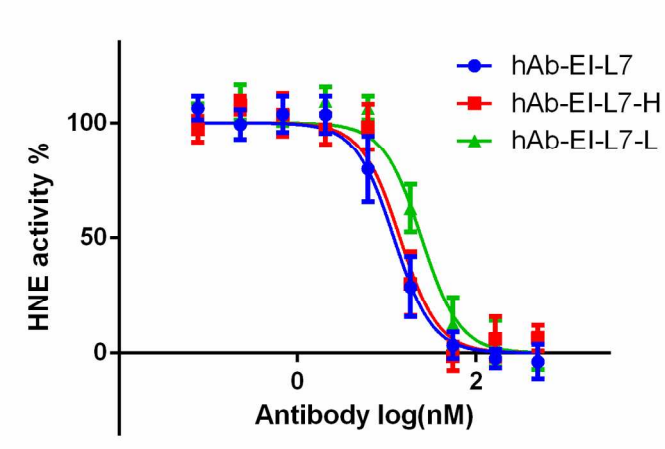

Figure S13. Activities of hAb-EI-L7 mutants. HNE at 20 nM was incubated with increasing concentrations of Fabs for 15 min, followed by the addition of 100 μM MeOSuc-AAPV-AMC. Residual enzyme activity was monitored using a plate reader (380 nm excitation and 460 nm emission). Assays were performed in triplicate.

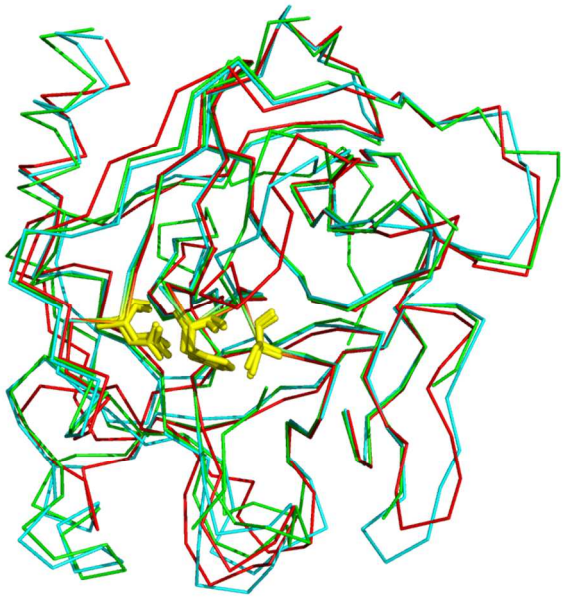

Figure S14. Backbone structure alignment of trypsin (cyan, PDB code 2PTC), chymotrypsin (green, PDB code 1GMD) and neutrophil elastase (red, PDB code 3Q76). Catalytic triads were labeled as yellow.

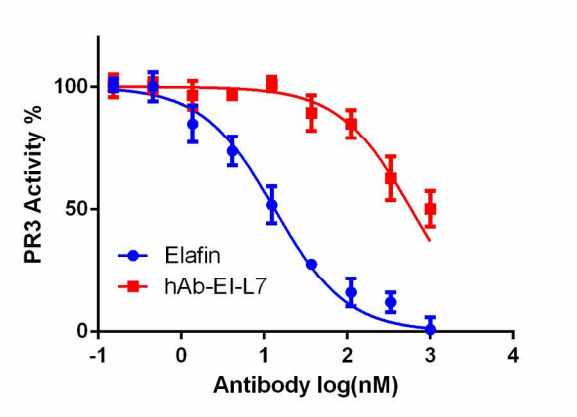

Figure S15. PR3 inhibition assay. 15 nM PR3 was incubated with increasing concentrations of hAb-EI-L7 and His-tagged recombinant elafin in PBS buffer. After 15 min incubation, 0.25 mM substrate Boc-Ala-Ala-Nva-SBzl and 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) in DMSO were added (with final DMSO concentration being 10%). Absorbance at 405 nm was recorded on a Molecular Devices plate reader. Assays were performed in triplicate.