Supplementary Data:
Experimental Procedures

Generation of prostasin expression constructs

Prostasin cDNA(PRSS8, NM_002773) was purchased from GeneCopeia. Site-directed mutagenesis was used to insert an enterokinase cleavage site between the propeptide and protease domains, and to change cysteine residues 154 and 203 to serine and alanine, respectively (GeneWiz). PCR was used to remove the N-terminal secretion signal and C-terminal GPI-anchor, and to add a C-terminal thrombin cleavage site. The product was then inserted into a restriction-digested pET28a(+) vector (EMD), carrying a C-terminal His_6 tag. Cloning of constructs for baculovirus expression was performed similarly to that for E coli, but the final vectors used were either pBAC-3 (EMD) (variants 801, 40) or pMelBac B (Invitrogen) (variant 35), and the propeptide was removed entirely. For variant 40, site-directed mutagenesis was also used to change asparagine 159 to serine (GeneWiz).

Prostasin expression and purification from insect cells. Generation of recombinant baculovirus using the flashBAC (NextGenSciences) system was performed using standard procedures. Prostasin variants were expressed in Sf-21 cells at an MOI=1 in SF-900 II media. The cell supernatants were harvested after 72 hours of growth at 27ºC. The pH of the supernatant was adjusted via addition of HEPES (50 mM) to a final pH of 7.5. The supernatant was centrifuged to remove any precipitation, concentrated 8 fold and diafiltered (2.5 diavolumes) against a buffer of 50 mM HEPES, 50 mM NaCl. Protein was then loaded onto a 80 mL Q Sepharose FF column (GE Healthcare) at 10 mL/min. Prostasin was then eluted in a buffer of 50 mM HEPES, pH 7.5 with a gradient from 50-350 mM NaCl over 30 column volumes. Fractions from all columns were evaluated using activity assays and SDS-PAGE. Pooled protein was loaded onto a 5 mL HisTrap Crude FF column (GE Healthcare) at 3 mL/min and was eluted in a buffer of 50 mM Tris/HCl, pH 8.5, 0.15 M NaCl, with a gradient from 15-300 mM imidazole over 50 column volumes. Pooled fractions were loaded on a 1 mL Benzamidine FF column, washed with 50 mM Tris/HCl, pH 8.5, 0.25 M NaCl, and eluted with 50 mM Tris/HCl, pH 8.5, 0.5 M NaCl, and 20 mM p-aminobenzamidine. Following a 10-fold dilution with 50 mM Tris/HCl, pH 8.5, the pooled protein was loaded onto a 1 mL QHP column (GE Healthcare) at 1 mL/min. Prostasin was then eluted in a buffer of 50 mM Tris/HCl, pH 8.5, with a gradient from 50-335 mM NaCl over 30 column volumes. The C-terminal 6 histidine tag was removed by cleavage with immobilized thrombin prior to kinetic analysis. For variant 40, the benzamidine column was replaced with a Superdex 200 column (1.6x120 cm), run in 50 mM Tris/HCl, pH 8.5, 50 mM NaCl. For variant 801, the Ni(II) column was omitted, and protein was bound in a batch mode to aprotinin-agarose (Sigma) in 50 mM Tris/HCl, pH 8.0. Prostasin was eluted from this material with 1 column volume of 0.1 M glycine, pH 3.0, which was immediately neutralized with Tris to pH 8.0. This variant was isolated with a truncated C-terminus due to nonspecific protease activity in the culture medium.

Expression of prostasin in E. coli. Prostasin variants 26 and 28 (Fig. 1) were transformed into BL21(DE3) competent cells (Novagen) via heat shock. Cells were grown at 37ºC in Superbroth to an optical density of 4-6 at 600 nm. Protein expression was then induced by addition of 0.4 mM IPTG for 3-4 hours. Cells were harvested by centrifugation and cell pellets stored at -80ºC until use.

Solubilization of E. coli pellets. Cell pellets from 5-10 L of cell culture were thawed and resuspended for 1 hour on ice at 5 mL/g pellet in a lysis buffer containing 25 mM Tris/HCl, 0.15 M NaCl, and 30% glycerol, pH 8.0 (Sigma) with the addition of 1x Bugbuster™, 5 U/mL Benzonase, and 750 U/mL lysozyme (Novagen). The slurry was briefly sonicated (6 times for 10 seconds each) to further disrupt cells. The lysate was centrifuged for 20 minutes at 25000 g.

The pellet from lysis was resuspended and centrifuged out of the following solutions: PBS + 1% Triton X-100 (2x), PBS (2x), and water. The final pellet was solubilized in 8 M urea with 0.1 M Tris/HCl, pH 8.0, and 2 mM DTT at an approximate concentration of 20 mg/mL (pellet wet weight). After assessing protein concentration via SDS-PAGE and Bradford assay, the solubilized pellets were then bound to Ni-NTA Superflow resin (Qiagen) at a final concentration of 2 mg/mL resin, and incubated as a slurry for 20 minutes prior to pouring out as a column. Following a wash with 2 bed volumes of 8 M urea, 0.1 M potassium phosphate, and 10 mM Tris, pH 8.0 buffer, and a second wash with 2 bed volumes of the same buffer with 15 mM imidazole, solubilized protein was then eluted with 1 bed volume of 8 M urea, 0.1 M potassium phosphate, 10 mM Tris, and 0.3 M imidazole pH 8.0.

Refolding and purification of prostasin zymogen. The urea-solubilized material was slowly pumped into a buffer containing 1 M L-arginine, 0.1 M Tris/HCl, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione, pH 8.0, to a final concentration of 10 mg/L. This was stirred overnight, and then diafiltered against a buffer containing 50 mM Tris/HCl, 50 mM NaCl, pH 8.0. After 3.5 volumes of diafiltration, the resultant solution was concentrated 3 fold. Protein was loaded onto a 5 mL HisTrap Crude FF column (GE Healthcare) at 3 mL/min and eluted in a buffer of 50 mM Tris/HCl , pH 8.5, 0.15 M NaCl, with a gradient from 15-300 mM imidazole over 50 column volumes. The eluted fractions were evaluated via SDS-PAGE and MS. Pooled fractions were diluted 3 fold with 50 mM Tris/HCl, pH 8.5 and loaded onto a 5 mL anion exchange column (QHP, GE Healthcare) at 5 mL/min. Prostasin was eluted in a buffer of 50 mM Tris/HCl, pH 8.5, with a gradient from 50-525 mM NaCl over 50 column volumes. The eluted fractions were evaluated via SDS-PAGE and MS, and fractions in which prostasin was detected by MS were pooled for further processing.

Cleavage and final purification of prostasin. Prostasin zymogen was converted to the active form by addition of enterokinase (EKMax, Invitrogen) at a final concentration of 2U/mL (7.5 U/mg prostasin). Reduced glutathione was added to a final concentration of 0.5 mM, and the resultant cleavage reaction was maintained at 4ºC for 48 hours and monitored by MS and SDS-PAGE. Following completion of cleavage as determined by MS, oxidized glutathione was added to a final concentration of 1 mM and the reaction incubated at 4ºC overnight. Protein was loaded onto a 5 mL HisTrap Crude FF column (GE Healthcare) at 3 mL/min and eluted in a buffer of 50 mM Tris/HCl, pH 8.5, 0.15 M NaCl, with a gradient from 15-300 mM imidazole over 20 column volumes. The eluted fractions were evaluated via activity assay, SDS-PAGE and MS. Pooled fractions were diluted 3 fold with 50 mM Tris/HCl, pH 8.5 and loaded onto a 1 mL QHP column (GE Healthcare) at 1 mL/min. Prostasin was eluted in a buffer of 50 mM Tris/HCl, pH 8.5, with a gradient from 50-335 mM NaCl over 30 column volumes. Fractions were selected based on activity and MS signature, then pooled to give the final purified prostasin for further characterization or crystallography.

Supplemental Tables

Supplementary Table 1. Mass spectrometric characterization of prostasin variant 28 during proteolytic activation.

	0.5 mM reduced glutathione treatment			
	-		+	
Protein	Mass	% of total	Mass	% of total
Zymogen	30,651	100	30,651	100
Cleaved	28,355 30,670	35 65	28,353	100
Cleaved + Nafamostat	28,350 28,515 30,670	20 15 65	28,515	100

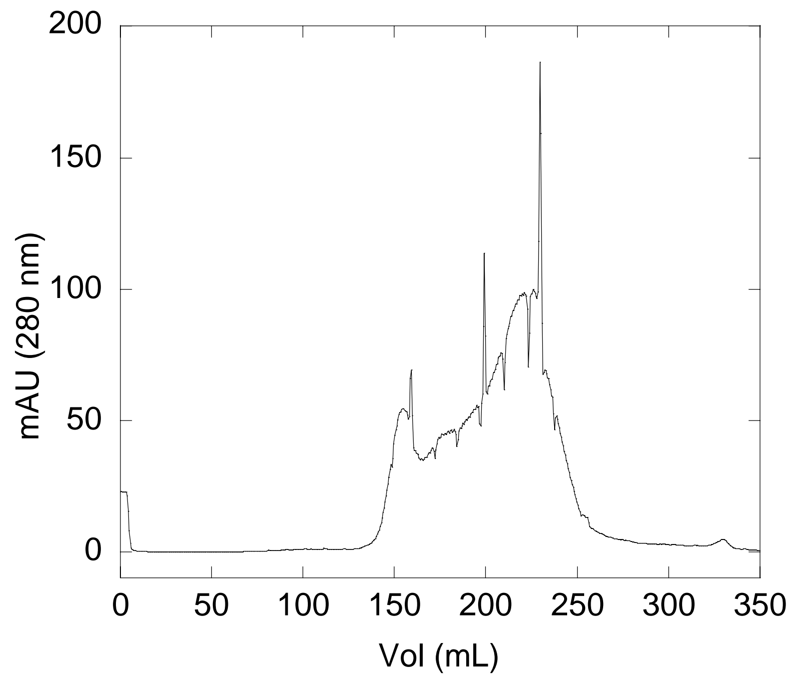
Table of mass species observed for prostasin variant 28 during enterokinase cleavage with and without glutathione treatment. Multiple species are observed without glutathione treatment, suggesting non-native disulfides are present, and only a minority of the protein is nafamostat reactive. Mass spectrometric measurements for each protein sample were made with a nominal 0.2-0.4 µg injection.

Supplementary Table 2. Analytical SEC data for prostasin variants.

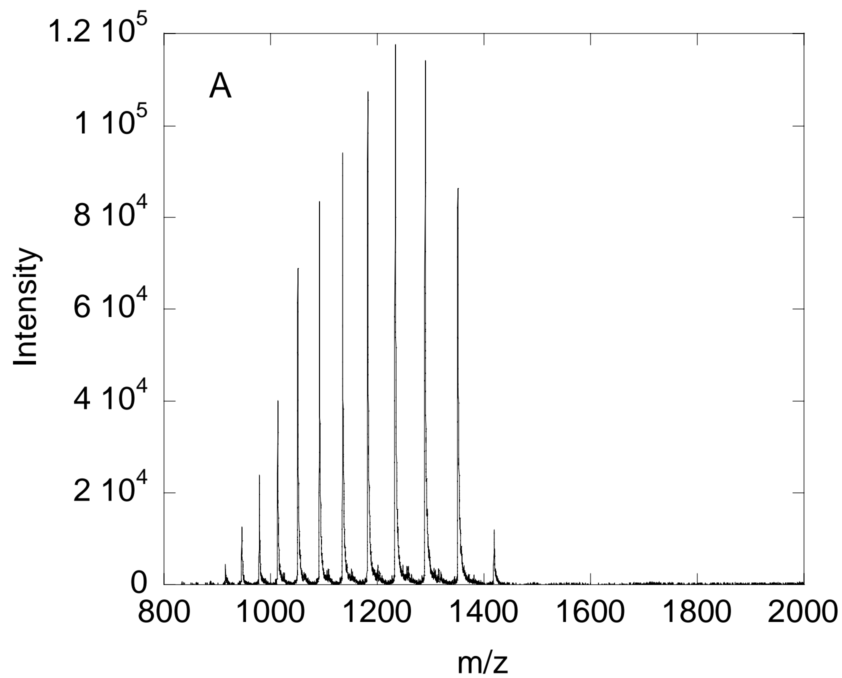
Variant	Retention time (min)	Apparent MW (kDa)
801	13.91	24.9
35	14.03	24.2
26	14.14	22.3
28	14.01	23.9

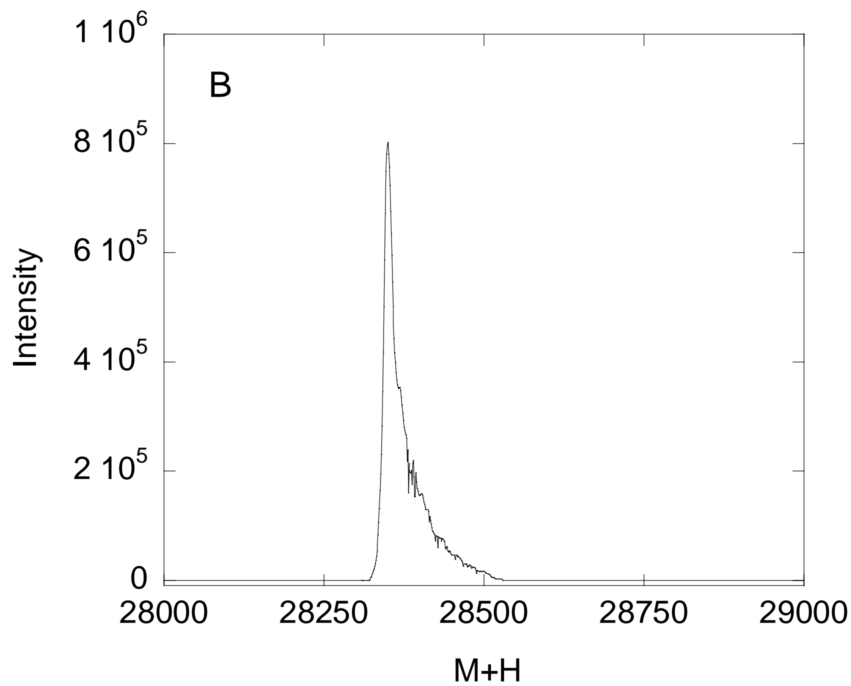
Table of retention times and apparent molecular weight for purified prostasin variants on analytical SEC. Data was obtained from injections of 1-5 µg of protein onto a 5x150 mm Superdex 200 column in a buffer of 50 mM potassium phosphate, pH 6.8, 0.3 M NaCl at a flow rate of 0.15 mL/min. Apparent MW was calculated by comparison to a set of mixed protein standards (Bio-Rad).

Supplemental Figures

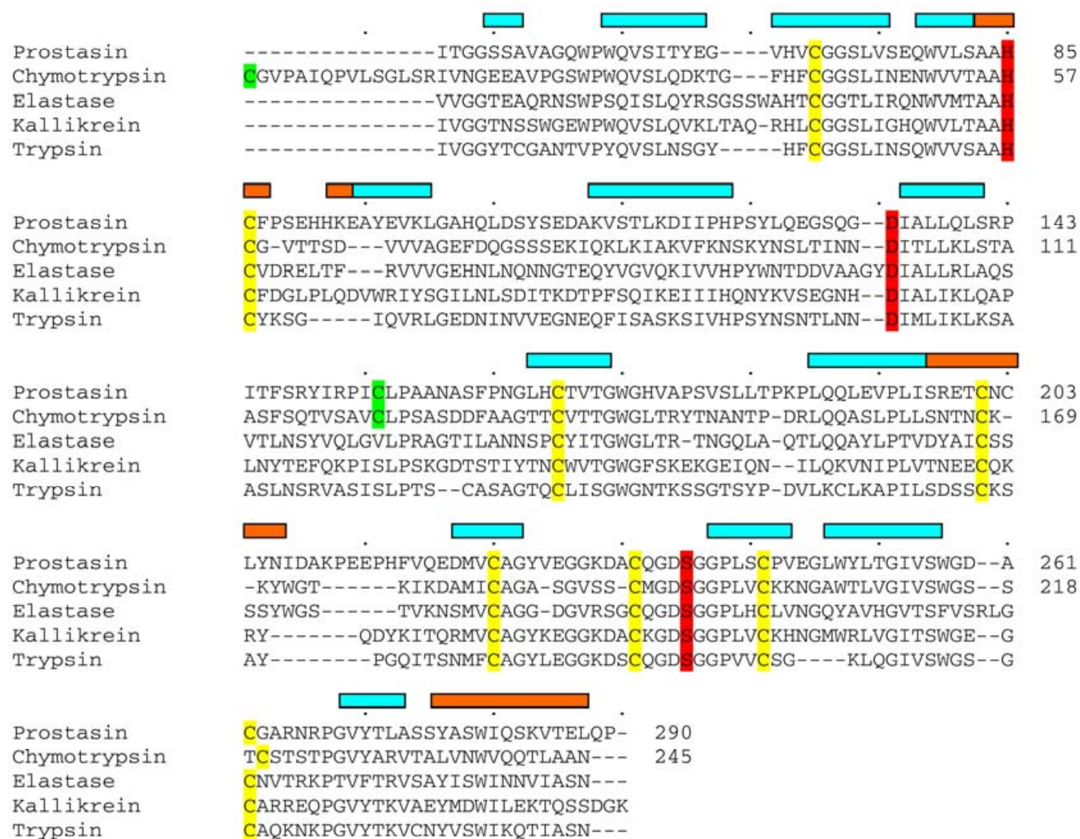


Supplementary Figure 1. Anion-exchange chromatographic trace of refolded prostasin 28 zymogen. The entire peak from ~140 mL to 250 mL contained prostasin zymogen as detected by SDS-PAGE and mass spectrometry.





Supplementary Figure 2: Mass spectrum of prostasin variant 28. A. Raw mass spectrum acquired as described in materials and methods section from 0.5 µg of final refolded protein. B. The deconvoluted mass spectrum of prostasin variant 28.



Supplementary Figure 3: Multiple sequence alignment between prostasin with other serine proteases. For reference, the sequence numbering for chymotrypsin and prostasin are shown. Residues of the catalytic triad are colored in red. Cysteines that form the four conserved disulfide bonds are colored in yellow. A fifth disulfide bond is formed in chymotrypsin between Cys1 and Cys122, which are colored green. Cysteine 154 of prostasin is also shown in green. Secondary structural domains of prostasin are shown above the sequence with helices in orange and sheets in blue. The sequence identity between the serine protease domain of prostasin and serine protease domains of chymotrypsin, trypsin, elastase, and kallikrein are 37%, 38%, 32%, and 39%, respectively.