Supplement Material : Material and Methods

Reagents

FXa, FX, and FIXa were from Enzyme Research Laboratories (South Bend, IN). DEGR-FXa, FXIa, FVa, prothrombin and thrombin were from Hematologic Technologies (Essex Junction, VT). Fibrillar collagen (from equine tendons) was from Chrono-log (Haverton, PA), and U46619 was purchased from Cayman Chemicals (Ann Arbor, MI). APTT (STA-PTT Automate) and PT (Neoplastine CI Plus) reagents were from Diagnostica Stago (Asnieres, France). S2222 (N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroaniline hydrochloride) and S2238 (H-d-phenylalanyl-l-pipecolyl-l-arginine-p-nitroaniline dihydrochloride) were obtained from Diapharma (West Chester, OH). Phosphatidylcholine and phosphatidylserine were from Sigma Co. (Saint Louis, MO). Anti-phospho-ERK 1/2 and polyclonal anti-ERK 1/2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated with biotin and peroxidase-conjugated streptavidin were obtained from Zymed (Invitrogen, San Diego, CA).

Sand Flies and Preparation of SG Homogenate (SGH)

L. longipalpis (Jacobina strain), *P. papatasi*, and *P.duboscqi* were reared at the Laboratory of Malaria and Vector Research (NIAD/NIH) using as larval food a mixture of fermented rabbit feces and rabbit food. Adult sand flies were offered a cotton swab containing 20% sucrose, and females were used for dissection of SGs at 4–7 days following emergence. SGs were stored in groups of 10 pairs in 10 μL NaCl (150 mmol l^{-1}), Hepes buffer (10 mmol l^{-1}; pH 7.4) at –70°C until needed. SGs were disrupted by ultrasonication within 1.5-mL conical tubes. Tubes were

centrifuged at 16,000×g for 5 minutes, and the resultant supernatant was diluted in PBS and used for the assays.

Lufaxin Properties

cDNA for mature Lufaxin (gi41397464; clone LJL143) codes for a protein of predicted molecular weight of 32495.78 da (278 amino acids [aa]) with an estimated pI 8.27. Extinction coefficient at 280 nm is 36180 (all disulfide bonds); A280 nm/cm$^{0.1\%}$ (1 mg/ml), 1.0975.

Cloning of *L. longipalpis* cDNAs in His-Tagged TOPO Vector

VR2001-TOPO is a topoisomerase adaptation of VR1020 plasmid (Vical, Inc., San Diego, CA) described in a previous report.[1] cDNA of Lufaxin (and other candidates) were amplified by PCR using a specific forward primer deduced from the amino-terminus region and a specific reverse primer containing an *ATGATGATGATGATGATG* motif between the stop codon and the carboxy-terminus region to introduce a 6xHis tag. The expected amplified sequences were predicted to code for proteins starting after the natural cleavage site and containing a 6xHis tag at the C-terminus region. PCR amplification conditions were: 1 hold of 94ºC for 5 minutes, 2 cycles of 94ºC for 30 seconds, 48ºC for 1 minute, 72ºC for 1 minute, 23 cycles of 94ºC for 30 seconds, 58ºC for 1 minute, 72ºC for 1 minute, and 1 hold of 72ºC for 7 minutes. Amplified products were extracted from a 1.0% agarose gel using Ultrafree-MC extraction kit (Millipore, Billerica, MA). Three μL of each PCR product was immediately incubated with 0.5 μL of VR2001-TOPO, 1 μL of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), and 1.5 μL H_2O for 5 minutes at room temperature. Transformation and selection of positive clones by sequencing were performed following standard procedures. [1]

Production and purification of recombinant proteins

VR2001-TOPO plasmids coding for Lufaxin (clone LJL143; gi 41397464,AY445936) and other salivary proteins (LJM17, AF132518; LJM04, AF132517; LJL15, DQ190946; LJM11, AY445935; LJL 13, AF420274; LJM111, DQ192488; LJM26, AY455913) were used for protein expression in HEK-293 F cells at the Protein Expression Laboratory at NCI-Frederick (Frederick, Maryland), and reported elsewhere.[2] All proteins were expressed containing a 6xHis tag. The supernatant was collected after 72 hours and concentrated from 500 ml to 300 ml using a Stirred Ultrafiltration Cell unit (Millipore) with a 30 kDa ultrafiltration membrane (Millipore). The volume was returned to 1 L by the addition of 500 mM NaCL and 10mM Tris, pH 8.0. The protein was purified by an HPLC system (DIONEX) using two 5ml HiTrap Chelating HP columns (GE Healthcare) in tandem and charged with 0.1 M $NiSO_4$. The protein was detected at 280nm and eluted by an imidazole gradient as follows: 0-5 min, 100% Buffer A (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH 7.4, 500 mM NaCl); 5-15 min, a gradient of 0% to 100% Buffer B (Buffer A+50 mM imidazole); 15-17 min, a gradient of 0% C (Buffer A + 500 mM imidazole) to 10% C (90% B); 17-22 min, 90% B and 10% C; min 22-27, a gradient of 10%C to 20% C (80% B); 27-35 min, 80% B and 20% C; 35-40 min, a gradient of 20% C to 100%C; and 40-50 min, 100% C. Eluted proteins were collected every minute in a 96-well microtiter plate using a Foxy 200 fraction collector (Teledyne ISCO). Fractions corresponding to peak(s) were selected and run on a NuPage Bis-Tris 4-12% Gel (Novex) with MES running buffer under reducing conditions as per manufacturer's instructions. Briefly, NuPage LDS sample buffer (Invitrogen), NuPage reducing agent (Invitrogen), and sample were combined and heated to 70˚C for 10 min. Samples were loaded in gel with SeeBlue Plus2 Pre-Stained Standard (Invitrogen) and run at 200V for 35 min with an expected current of 100-125mA (start), 60-

80mA (end). After run, gel was stained with Comassie Blue (0.025%) to visualize proteins. Appropriate fractions as determined by molecular weight compared to standard in gel were pooled and concentrated to 1ml using a 30kDa Amicon Ultra Centrifugal Filter (Millipore). The protein sample was then injected into a g2000sw molecular sieving column (Tosoh Biosciences) with a 1 ml loop connected to HPLC (DIONEX) with PBS pH 7.2 as the buffer for further purification. The protein was detected at 280 nm and the fractions were collected as described above. Appropriate fractions were determined as described above and pooled. Concentration was measured by using a NanoDrop ND-1000 spectrophometer at 280 nm and calculated using the extinction coefficient of the protein.

Polyclonal Antibodies against Lufaxin

VR2001-TOPO plasmids containing coding sequence of Lufaxin without His-tag extension were used to inject mice (20 μg, intradermally in the ear of mice) and generate polyclonal antibodies. Pre-immune samples were drawn before the first injection, and immune serum samples were taken by retroorbital bleeding after 3 injections given at 2-week intervals. Each mouse serum sample was pooled for experimentation.

PAGE and Western Blotting

The samples were treated with 4× NuPAGE LDS sample buffer and analyzed in NuPAGE 4–12% gels with MES running buffer. SeeBlue® Plus2 molecular weight marker was used. The proteins in the gel were transferred to nitrocellulose membrane using an iBlot™ device. After blocking with 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), pH 8.0, the membrane was incubated with sera of mice immunized with Lufaxin (1:100 in TBS-T 5 % milk).

After two washes with TBS-T, the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (ZyMax^{TM}) at 1:10000 in TBS-T 5 % milk for 40 minutes at room temperature. After 3 washes with TBS-T, the blots were developed by addition of Western Blue® stabilized substrate for alkaline phosphatase (Promega). For N-terminal sequening, Lufaxin was loaded in a 4-12% NU-PAGE and transferred to PVDF membranes (0.45 μM) in 10 mM CAPS, 10% methanol, pH 11. All non-specified reagents were from Invitrogen.

Deglycosylation of Lufaxin

This was performed using the Enzymatic DeGlycoMx Kit from QA-Bio (Palm Desert, CA) which contains a mixture of PNGase F, Sialidase, ß-Galactosidase, Glucosaminidase and O-Glycosidase. The assay was performed following the manufacturer instructions. Briefly, in a PCR tube, 5 μl of Lufaxin (5 μg) was added to 33 μl of water, followed by addition of 2.5 μl of denaturing solution. The samples were mixed and heated at 99^{o}C for 10 min. Then, 2.5 μl of Triton-X was added, followed by addition of 2 μl of DeGlycoMx for 3 hr at 37^{o}C. Samples were analyzed by NU-PAGE, and Coomassie Blue stained.

Platelet Aggregation Assays

Platelet-rich plasma was obtained by plateletpheresis from medication-free platelet donors at the DTM/NIH blood bank. Aggregation was performed as described previously.[2]

Contraction of Rat Aorta

Contraction of rat aortic ring preparations by U 46619 was measured isometrically and recorded with transducers from Harvard Apparatus Inc. (Holliston, MA) as reported.[3] A modified Tyrode

solution was prepared with the addition of 5 mM Hepes; the pH was adjusted to 7.4, and the solution was oxygenated by continuous bubbling of air throughout the assays. In the first assay, aortic rings were suspended in a 0.5-mL bath kept at 36°C and were pre-constricted by 100 nM U-46619 before addition of Lufaxin to give final concentrations of 1 μM, or salivary gland homogenates of *Rhodnius prolixus* (0.04 of one pair of glands/ml, with an approximate final concentration of nitrophorins of 2 μM - positive control). Additions to the bath were never greater than 5% of the volume of the bath.

Recalcification Time

Clotting activity was measured by the recalcification time of human plasma using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA) with a kinetic module. Briefly, 30 μL of citrated human platelet-poor plasma and 30 μL of sample (SGH or recombinant Lufaxin diluted in TBS) were mixed in 96-well flat-bottom plates. After incubation of 10 minutes at 37^{o}C, 30 μL of pre-warmed 25 mM $CaCl_2$ (final concentration 7.5 mM, diluted in TBS) were added using an 8-channel multipipetter for delivering calcium aliquots. The plate was immediately mixed and maintained at 37°C during the kinetic experiment. Absorbance readings at 650 nm were taken at 10-second intervals for 20 minutes. Onset time was defined as the time to a linear increase in the OD, which reflects the maximal rate of formation of insoluble fibrin set at a OD which is approximately half of the maximum obtained in the presence of calcium only (no inhibitor).

aPTT and PT Assays

The effect of Lufaxin on coagulation tests aPTT and PT was evaluated on an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany). Human blood samples were collected from

healthy donors in 3.8% trisodium citrate (9:1, v/v), and platelet-poor plasma was obtained by centrifugation at 2,000×g for 10 minutes. Mouse plasma samples were obtained following the same procedure after blood collection by cardiac puncture. Plasma (50 μL) was incubated with Lufaxin for 2 minutes at 37ºC, followed by addition of the aPTT reagent (50 μL, 1 minute) or the PT reagent (100 μL) and then 25 mM $CaCl_2$ (100 μL). Time for clot formation was then recorded. For ex vivo assays in mice, phosphate-buffered saline (PBS) or Lufaxin were given i.v. 15 minutes before cardiac puncture. Blood collection, plasma preparation, and aPTT procedure were performed as above.

Prothrombinase Assembly

Activation of prothrombin by human FXa was performed in TBS-Ca^{2+} (20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.3% BSA, pH 7.5), using a discontinuous assay.[4] FXa (20 pM, final concentration), was incubated with Lufaxin (0-10 nM) for 20 minutes at room temperature. Human FVa (1 nM, final concentration) and PC/PS vesicles (10 μM, final concentration) were added and incubated for 5 minutes. Reactions were initiated by addition of human prothrombin (1.4 μM, final concentration). Aliquots of 25 μL were removed every minute into microplate wells containing 50 μL of TBS-EDTA (20 mM Tris-HCl, 150 mM NaCl, 20 mM EDTA, 0.1% BSA, pH 7.5) to stop reactions. After addition of 25 μL of S-2238 (312.5 μM), absorbance at 405 nm was recorded at 37°C for 15 minutes at 11-second intervals using a Thermomax microplate reader (Molecular Devices).[5] Initial velocities (Vmax mode, mOD/minute) obtained were used to calculate the amount of thrombin formed, using a standard curve. Absence of one of the components in the prothrombinase showed no thrombin formation.

Interaction of Lufaxin with phospholipid vesicles

Lufaxin (1.25 μM) was incubated with PC/PS (10 μM) in 10 mM TBS (pH 7.4) supplemented with $CaCl_2$.After 30 min, the mixture was centrifuged and NUPAGE sample buffer was added to the pellet and to the supernatant. The samples were loaded in a 4-12% NUPAGE gel, and Coomassie blue stained. Lufaxin which has not been incubated with PC/PS was used as control.

Kinetics Studies

This was performed as described [5] using chromogenic substrate (S2222) hydrolysis specific for FXa. Substrate hydrolysis was estimated by color development at 405 nm at room temperature, using an ELISA reader (Molecular Devices). All reagents were diluted in the reaction buffer, TBS-BSA (10 mM Tris, 0.15 M NaCl, 0.3% BSA, pH 7.4). To characterize the interaction of Lufaxin and FXa as tight, reactions were started by addition of S2222 (250 μM) to a mixture containing enzyme (2, 4 and 8 nM) and inhibitor (0-60 nM) pre-incubated for 1 hour at room temperature. The total volume of the reaction was 100 μL, and reactions were followed for one hour. Initial velocities (Vmax mode, mOD/minute) obtained were used to determine the ratio of substrate hydrolysis by *Vs*(inhibited)/*Vo*(uninhibited) by the enzyme in the presence of the inhibitor, which allows determination of the IC_{50}. [5, 6] For assays designed to determine whether Lufaxin is a slow or fast inhibitor of FXa, and the type of inhibition (competitive or non-competitive), the inhibitor (0-60 nM) was incubated with S2222 (150, 300, 450, 600, 750 and 900 μM) at room temperature for 5 minutes followed by addition of FXa (0.5 nM). Reactions were followed for 2 hours, in a 200 μL volume. In all kinetic measurements, care was taken to ensure that substrate was less than 20% hydrolyzed. The linear part of the progress curves between 45-60 min was chosen to determine the steady state kinetics of Lufaxin-FXa complex

formation. These values were used to plot *Vs*/*Vo* versus Lufaxin for each S2222 concentration, at a constant FXa concentration. Data points were fitted with the Morrison equation using GraphPad Prism software, which allows the determination of IC_{50} for each S2222 concentration:[5, 6]

$$Vs/Vo=\{[E_t]-[I_t]-Ki^*)+([I_t]+Ki^*-[E_t])^2+4Ki^*[E_t]^{1/2}\}/2[E_t]$$

where E_t is the total enzyme concentration, I_t is the total inhibitor concentration and Ki^* is the IC_{50} or apparent Ki. The plot IC_{50} vs S2222 was then used to calculate the type of inhibition and true Ki. Data points are the mean of 6 determinations, each performed in duplicates. In some experiments to determine the role of Ca^{2+} or phospholipids in Lufaxin interaction with FXa, Ca^{2+} (0.5 or 5 mM), or PC/PS vesicles (20 μM) was added to the reaction mixture.

Determination of the active site of Lufaxin

In a PCR tube, Lufaxin (1.9 μM) was incubated with human FXa (2.7 μM) for 3 or 24 hours at 37^{o}C, with and without 5 mM Ca^{2+}. LDS loading buffer and DTT was added to the tubes, warmed for 10 min at 70^{o}C, and the mixture was loaded in a 4-12% NU-PAGE gel (MES buffer) and Coomassie Blue stained. See Blue standard was used as molecular weight marker (Invitrogen).

Surface Plasmon Resonance (SPR)

All SPR experiments were carried out in a T100 instrument (Biacore Inc., Uppsala, Sweden) following the manufacturer's instructions. For immobilization using an amine coupling kit (Biacore), CM5 chips were activated with 1-ethyl-3-(dimethylaminopropyl) carbodiimide, and N-hydroxysuccinimide before injection of Lufaxin (6.5 μg/mL) in acetate buffer, pH 5.5.

Remaining activated groups were blocked with 1 M ethanolamine, pH 8.5, resulting in a final immobilization of 604.5 RU. Kinetic experiments were carried out by injecting FXa for a contact time of 180 seconds at a flow rate of 30 µL/minute at 25°C. For all runs, HBS-P buffer was used (10 mM HEPES, 150 mM NaCl, 0.005% surfactant P20, pH 7.4). FXa-Lufaxin complex dissociation was monitored for 600 seconds, and the sensor surface was regenerated by a pulse of 30 seconds of 10 mM HCl at 30 µL/minute. In some experiments, other coagulation factors (thrombin, FXIa, FIXa, DEGR-FXa, or FX) were tested (100 nM) as analytes. For some experiments, FXa (30 µg/mL in acetate buffer pH 5.0) was immobilized in CM5 sensor chips at 661.7 RU, and SGH from *L. longipalpis* was used as analyte. Blank flow cells were used to subtract the buffer effect on sensorgrams. After subtraction of the contribution of bulk refractive index and nonspecific interactions with the CM5 chip surface, the individual association (*ka*) and dissociation (*kd*) rate constants were obtained by global fitting of data using the 1:1 model (Langmuir) interaction model using BIAevaluation™ (Biacore, Inc.). Values were then used to calculate the equilibrium constant (*KD*). The values of average squared residual obtained were not significantly improved by fitting data to models that assumed other interactions. Conditions were chosen so that the contribution of mass transport to the observed values of *KD* was negligible. Also, models in the T100 evaluation software fit for mass transfer coefficient to mathematically extrapolate the true *ka* and *kd*.

Isothermal Titration Calorimetry (ITC)

Lufaxin binding to FXa was performed using a VP-ITC microcalorimeter (Microcal, Northampton, MA) at 30°C. Prior to the run, the proteins were dialyzed against 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4 for binding experiments. Titration experiments were performed by

making successive injections of 5 µL each of 20 µM FXa into the 2-mL sample cell containing 2 µM Lufaxin until near-saturation was achieved. The calorimetric enthalpy (ΔHcal) for each injection was calculated after correction for the heat of Lufaxin dilution obtained in control experiments performed by titrating Lufaxin into buffer. The binding isotherms were fitted according to a model for a single set of identical binding sites by nonlinear square analysis using Microcal Origin software (Microcal, Northampton, MA). The enthalpy change (ΔH) and stoichiometry (n) were determined according to equation 1:

$$Q = n\theta M_t \Delta H Vo \qquad (1)$$

where Q is the total heat content of the solution contained in the cell volume (Vo), at fractional saturation θ, ΔH is the molar heat of ligand binding, n is the number of sites, and Mt is the bulk concentration of macromolecule in Vo. The binding constant, Ka, is described as in (2):

$$Ka = \theta/(1-\theta)[X] \qquad (2)$$

where [X] is the free concentration of ligand.

The free-energy (ΔG) and entropy term (–TΔS) of association were calculated according to (3) and (4):

$$\Delta G = -RT\ln(Ka) \qquad (3)$$

$$\Delta G = \Delta H - T\Delta S \qquad (4)$$

Cell Culture

MDA-MB-231 breast cancer cells were maintained in ISCOVES medium (Invitrogen, CA) supplemented with 10% FBS in culture flasks in a 5% CO_2-air mixture at 37ºC. Subconfluent cultures were washed twice with PBS, and cells were detached with Hank's solution containing

10 mM HEPES and 0.2 mM EDTA. Cells were seeded at 5×10^5 cells/well in 6-well tissue culture plates for signaling assays.

PAR2 Signaling Assay

MDA-MB-231 cells were serum starved for 90 minutes and stimulated with 10 nM FXa (in ISCOVES medium, no FBS) for 10, 15, 30, and 60 minutes. Lufaxin (50 nM) was added 1 hour prior to stimulation with FXa. To avoid PAR1 activation by thrombin, assays were performed in the presence of 10 nM hirudin as described.[7] After the incubation period, cells were washed with PBS and lysed in cold buffer containing a phosphatase inhibitor cocktail (Sigma). Cell lysates (15 μL) were separated by SDS-PAGE (10%). Proteins were transferred onto polyvinylidene fluoride membranes (Millipore) and blocked with TBS supplemented with 5% BSA and 0.1% Tween 20 for 1 hour at room temperature. Membranes were then probed with primary antibody anti-pERK (1:000 in TBS, 2% BSA, 0.1% Tween 20, TBS-BSA) overnight at 4ºC. The membranes were washed 3 times with TBS-BSA before the addition of goat anti-mouse biotin-labeled secondary antibody (1:20,000 for p-ERK; 1:30,000 for ERK) for 1 hour at room temperature. The membranes were washed in TBS-BSA and probed with peroxidase-conjugated streptavidin (1:30,000; Zymed-Invitrogen, CA) for 1 hour at room temperature. After washing the membranes, immunodetection was carried out side-by-side by a chemiluminescent method using the Western Lightning ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and and Amersham Hyperfilm ECL (General Electric, UK). The blots were quantified by Scion Image software (Scion Corporation, USA). The relative levels of pERK were estimated as a ratio to ERK 1/2.

Paw Edema in Mice

Female C57BL/6 mice, 6–8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the NIAID Animal Care Facility. [8] Mice were maintained at an American Association of Laboratory Animal Care-accredited facility at NIAID, National Institutes of Health. All experiments with mice were evaluated and approved by the NIAID Animal Care and Use Committee of the National Institutes of Health (Rockville, MD). Prior to each injection, the posterior footpad thickness of each mouse was recorded using a caliper (Mitutoyo America Corp., Aurora, IL). Subsequently, 30 μL of PBS, or 30 μL of FXa (7.3 μM, 10 μg), or 30 μL of a mixture containing 30 μL FXa (7.3 μM) plus Lufaxin (2.4 μM), or 30 μL FXa (7.3 μM) plus Lufaxin (9.8 μM) were injected intradermally in the paw using a 0.3-mL syringe U-100 29g1/2 (ref 309301) from Becton Dickinson (Franklin Lakes, NJ). As an index of edema formation, paw thickness was then measured at 15, 30, 45, and 60 minutes. Control groups of mice received the same volume of PBS (vehicle). For each data point, 4 posterior paws were injected. Statistical analysis of variance using Tukey as a multiple comparison post-test was used. A p value of 0.05 or less was considered statistically significant.

$FeCl_3$-Induced Artery Thrombosis

BALB/c mice were anesthetized with intramuscular xylazine (16 mg/kg) followed by ketamine (100 mg/kg). The right common carotid artery was isolated through a midline cervical incision, and blood flow was continuously monitored using a 0.5 VB Doppler flow probe coupled to a TS420 flow meter (Transonic Systems, Ithaca, NY). Fifteen minutes before induction of thrombosis, animals were injected in the tail vein with 50 μl Lufaxin (0.2 or 0.5 mg/kg) or vehicle. Thrombus formation was induced by applying a piece of filter paper (1 × 2 mm)

saturated with 7.5% $FeCl_3$ solution on the adventitial surface of the artery for 3 minutes. After exposure, the filter paper was removed, and the vessel was washed with sterile normal saline. Carotid blood flow was continuously monitored for 60 minutes or until complete occlusion (0 flow for at least 10 seconds) occurred. Statistical analysis of variance using Tukey as a multiple comparison post-test was used. A *p* value of 0.05 or less was considered statistically significant.

Tail Bleeding Assay

Mice were anesthetized as described above and injected intravenously with PBS or Lufaxin in a 100-μL volume. After 15 minutes, the distal 2-mm segment of the tail was removed and immediately immersed in a 50-mL Falcon tube filled with saline buffer warmed to 37ºC. The samples were properly homogenized and the absorbance determined at 540 nm to estimate hemoglobin content. No animal was allowed to bleed for more than 30 minutes.

Protease inhibition assays

All assays were performed at 30^{o}C in triplicates. One hundred nM of Lufaxin was pre-incubated with each enzyme for 20 min before the addition of the corresponding substrate. Hydrolysis rate of the fluorescent substrate was estimated from the slope that results from the linear fit (arbitrary fluorescence units per sec; $\chi 2 > 0.95$) of the data (each experiment was performed in triplicate, and the mean of the three experiments and the standard error of the mean were calculated). The linear fit of the fluorescence increase as a function of time was verified with the Magellan™ - Data Analysis Software (Tecan group Ltd). The observed substrate hydrolysis rate in the absence of protein was considered as 100% and compared with the remaining enzymatic activity in the presence of the protein.

All enzymes used were of human origin, purified or recombinant. The source and assay concentration of the different enzymes follow; thrombin (0.01 nM), α-chymotrypsin (0.04 nM), plasmin (0.8 nM), and chymase (0.45 nM) were purchased from Sigma; tryptase (0.01 nM) was purchased from Promega; FXa (0.5 nM)was purchased from EMD Biosciences (Madison, WI); FXIIa (0.15 nM) was purchased from Haematologic Technologies Inc.; kallikrein (0.06 nM) was purchased from Fitzgerald Industries International ; elastase (0.18 nM) was purchased from Elastin Products; cathepsin G (4.4 nM), FXIa (0.06 nM), uPA (0.125 nM), and tPA (0.025 nM) were from Molecular Innovations; matriptase (0.04 nM) was from R&D Systems; proteinase 3 (5.5 nM) was from Merck; and sequencing-grade trypsin (0.2 nM) was purchased from Roche. Assay buffers were: for elastase, proteinase 3 and chymase, 50 mM Hepes buffer, pH 7.4, 100 mM NaCl, 0.01% Triton X-100; for trypsin and chymotrypsin, FXIa and FXIIa, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM $CaCl_2$, 0.01% Triton X-100; for thrombin, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.01% Triton X-100; for tryptase, 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.05% Triton X-100; for kallikrein, matriptase, and plasmin, 20 mM Tris–HCl, pH 8.5, 150 mM NaCl, 0.02% Triton X-100; for FXa, 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA; for uPA and tPA, 20 mM Tris-HCl, pH 8.5, 0.05% Triton X-100; and for cathepsin G, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Triton X-100. The substrates used were Suc-Ala-Ala-Pro-Val-AMC for elastase and proteinase 3, Boc-Asp-Pro-Arg-AMC for thrombin and plasmin, Boc-Gln-Ala-Arg-AMC for trypsin, factor XIa and uPA (Sigma), Boc-Phe-Ser-Arg-AMC for tryptase, Suc-Leu-Leu-Val-Tyr-AMC for chymase (Bachem Bioscience, Inc.), Suc-Ala-Ala-Pro-Val-AMC for chymotrypsin (EMD Biosciences) and methylsulfonyl-D-cyclohexylalanyl-Gly-Arg-AMC acetate for FXa, FXIIa, t-PA, matriptase, and kallikrein (American Diagnostica Inc.). All substrates were used in 250 μM final concentration in all

assays. Substrate hydrolysis rate was followed in a Tecan Infinite M200 96-well plate fluorescence reader (Tecan group Ltd, Switzerland) using 365 nm excitation and 450 nm emission wavelengths with a cutoff at 435 nm. *t*-test was used for statistical analysis and a *p* value of 0.05 or less was considered statistically significant.

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