Supplemental Methods and Figures

Mapping the Prothrombin Binding Site of Pseutarin C by Site-directed PEGylation

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Supplemental Methods

Stable expression of *P. textilis* fX and human prothrombin in HEK-EBNA cells and barium chloride precipitation

Cells expressing *P. textilis* fX and human prothrombin were grown in CD-CHO media supplemented with 4 mM L-glutamine, geneticin (G418, 0.025 mg/ml), hygromycin B (200 µg/ml) and vitamin K1 (10 µg/ml), and harvested every 2 to 3 days. Harvested media was pooled and concentrated 10-fold using a Vivaflow 200 (Sartorius) concentrator. Trisodium citrate was added to the concentrated media to a final concentration of 15 mM, followed by stirring for 30 minutes at 4°C. Cold 1 M barium chloride (80 mL/L media) was added slowly to effect precipitation of Gladomain proteins, with stirring for an additional 30 minutes and stationary incubation for 1 hour at 4°C. Precipitate was then pelleted by centrifugation at 6000 x g for 15 minutes at 4°C. The recovered pellets were dissolved in 0.2 M EDTA (150 mL/L media), followed by dialysis against 5 L 20 mM Tris pH 7.4, 50 mM NaCl, 1 mM EDTA and 1 mM Benzamidine-HCl overnight at 4°C. Dialysates were filtered before purification.

Purification of *P. textilis* fXa

Post dialysis, filtered *ptFX* was applied onto a HiTrap Q Sepharose column (5ml, Cytiva) equilibrated with 20 mM Tris pH 7.4, 50 mM NaCl and eluted with a gradient from 0.05-0.7 M
NaCl over 7 column volumes (CV). The recovered protein was then diluted 10-fold in 20 mM imidazole pH 6.5, 5 mM CaCl2 and loaded onto a heparin Sepharose column (5 ml, Cytiva) equilibrated with the same buffer. The protein was eluted with a gradient of 0-0.75 M NaCl over 10 CV. 5 µl from each fraction were added into a 96-well plate containing 45 µl Tris-Buffered Saline (TBS) with 5 mM CaCl2, activated with Russell's viper venom factor X activator (RVV-X, 1:100 w/w) and used in a factor Xa activity assay. Fractions capable of hydrolyzing S-2222 were pooled and concentrated for further purification on a Superdex 200 (PG 16/600, Cytiva) column equilibrated with 20 mM Tris pH 7.4, 150 mM NaCl and 5 mM CaCl2. RVV-X (1:100 w/w) was added to the purified ptfX and incubated at 37°C for 3h. Activated ptfX (ptfXa) was then applied to a heparin Sepharose column (5ml, Cytiva) and eluted as described above. Protein was concentrated (2 mg/ml) and dialyzed into 20 mM Tris pH 7.4, 150 mM NaCl and 5 mM CaCl2 buffer. Purity was assessed by SDS-PAGE analysis.

**Purification of human prothrombin**

Recombinant human prothrombin recovered after barium chloride precipitation was applied onto a Q Sepharose column (5ml, Cytiva) equilibrated with 20 mM Tris pH 7.4, 50 mM NaCl and eluted with a gradient from 0.05-1 M NaCl over 10 CV. Fractions were diluted 10-fold in 20 mM Tris pH 7.4, 1 mM EDTA and loaded on to a Blue Sepharose column (5 ml, Cytiva) equilibrated with 20 mM Tris pH 7.4, 1 mM EDTA, 100 mM NaCl. Elution was performed initially with a gradient from 0.1-2 M NaCl over 5 CV, which is then followed by a gradient from 2-4 M NaCl over 1 CV. Samples from fractions (2 µl) were added into 96-well plate containing 47 µl TBS and activated to thrombin to determine those containing prothrombin. PMSF-inactivated E. Carinatus venom (1 µl, 1 mg/ml) was added to each well and incubated for 60 minutes at room temperature, and
thrombin activity was monitored by addition of 50 µl of 0.4 mM S-2238 (Chromogenix) and reading the absorbance at 405nm using a Versamax plate reader (Molecular Devices). Fractions resulting in thrombin activity were pooled, concentrated and purified on a Superdex 200 (PG 16/600, Cytiva) column equilibrated with 20 mM Tris pH 7.4, 150 mM NaCl and 5 mM CaCl2.

**SDS-PAGE analysis for prothrombin processing**

Reaction mixtures containing 5 µM prothrombin, 20 µM DAPA, and 50 nM ptfV variants were added in assay buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2 mM CaCl2, 0.1% PEG8000) and reactions were initiated by the addition of 1 nM full-length ptfXa. Samples (10 µL) were withdrawn at time points and immediately quenched by mixing with 5 µL SDS sample buffer containing 100 mM DTT and 100 µM AEBSF and heating at 95°C for 5 minutes. Samples were run on 4-12% BisTris gradient gels (Invitrogen, Thermo Fisher Scientific) with MOPS buffer (Formedium). Bands were visualized by staining with Quick Coomassie Stain (Generon) at room temperature for 1 hour. Gels were destained and then imaged on a ChemiDoc MP Imaging System (Bio-Rad). Densitometry analysis was performed using Image Lab software (Bio-Rad).
Supplemental Figure 1. Labelling *P. textilis* fV variants with Maleimide-PEG40K. In order to assess labelling efficiency, each single Cys variant was reacted with Maleimide-PEG40K and run on SDS-PAGE (3-8% Tris-acetate gel, first and second gel) (A and B). Variants were first reduced with Pierce Immobilized TCEP disulfide reducing gel (Thermo Scientific) at room temperature for 45 minutes before reacting with 20-fold molar excess of Maleimide-PEG40K (Merck) at 4°C for 2 hours. Bands were visualized following staining with Quick Coomassie Stain (Generon) at room temperature for 1 hour. SDS-PAGE gels were destained and then imaged on a ChemiDoc MP Imaging System (Bio-Rad). Percent labeled was calculated with values obtained from densitometry. PtfV_C540A, used as a background for introduction of Cys mutations, was also incubated with Maleimide-PEG40K. No labelling was observed (C).
Supplemental Figure 2. Assessment of ability of Streptavidin mutein column to separate labelled from unlabelled ptfV. Wild-type *P. textilis* fV with a single free cysteine residue (C540, *ptfV* _C540_) was reacted with Maleimide-PEG11-Biotin and purified on a Streptavidin mutein column according to the manufacturer’s instructions, collecting flow-through and washes. Wash/Equilibration buffer contained 100mM Potassium Phosphate pH7.2, 150mM NaCl, 600mM Ammonium Sulphate, 0.1% PEG 8000 and protein eluted with buffer contained 100mM Potassium Phosphate pH7.2, 150mM NaCl, 2mM D-Biotin (A). (B) Purified fractions were run on SDS-PAGE and stained with Coomassie (left) and by Western Blotting using Streptavidin-HRP to assess separation of labelled and unlabeled fV.

Supplemental Figure 3. Introduction of the human fV and fVIII a1-loop sequences into *P. textilis* fV by a Klenow based ligation-independent technique. Primer sequences containing 24 bp overlap are shown.