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Recombinant PNGaseF production and deglycosylation assays.

Summary. 1.5 L of BL21 *E.coli* cells harbouring the pOPH6:*Em*PNGaseF vector encoding a His-tagged construct of *Elizabethkingia miricola* PNGase F (*Em*PNGaseF, Uniprot P21163, PNGF_ELIMR) were used to express the protein. *Em*PNGAse F was purified from the *E.coli* lysate by immobilised metal affinity and size exclusion chromatography and its activity assayed against 5 distinct glycoproteins.

Protein expression Two aliquotes of 5 mL of autoclaved low-salt LB (Sigma) made 100 µg/mL ampicyllin in 15 mL Falcon tubes were inoculated with 5+5 µL of BL21 *E.coli* cells (frozen in 20% glycerol and kept at -80 °C) harbouring the pOPH6:*Em*PNGaseF vector (courtesy of Dr. Dominic Alonzi and Dr. Sarah Needs). The *Em*PNGaseF gene in this vector bears a C-term HexaHistidine tag and an N-term OmpA3 leader that directs to the periplasm. The cells were left shaking at 200 rpm at 37 °C overnight and in the morning each volume was transferred to 720 mL of LB made 100 µg/mL ampicyllin - in two 2 L baffled flasks, the medium preheated to the same temperature.

Between 13:00 and 18:00 the cells grew from an optical density OD_{600} of 0.02 to 0.56, at which point each flask of cells was induced with 1 mM IPTG and the temperature set to 30 °C and left overnight shaking at 200 rpm.

Protein purification. In the morning after 16 hours expression the 1.5 L of cells were centrifuged in 0.5 L bottles at 8,000 g for 30' at 4 °C and the pellet freeze-thawed at -20 °C and then resuspended in 20 mL of lysis buffer: 0.5 M Sucrose, 0.1 M Tris pH 8.0 and a few milligrams of DNAse and Lysozyme (Sigma) on ice. The resuspended cells were sonicated on a Soniprep 150 sonicator (MSE) at power/amplitude setting 14: 6 alternations of 30" sound burst and 30" pause, for 3' total of sonication in a 6' minutes experiment.

The lysate was spun at 30,000 g for 30' and the 20 mL S/N from this spin mixed with 80 mL of binding buffer A: 2 mM Imidazole, 20 mM HEPES pH 7.6 and 300 mM NaCl. After filtering with a 0.22 µm filter, the S/N was passed on a 1 mL HisTrap Ni++ column (GE Healthcare) at about 3 mL/min. The column was then washed with 20 column volumes (CV) of buffer A and the protein eluted with a 20 CV gradient from buffer A to buffer B (B: obtained from A with the addition of 500 mM imidazole, re-pH'ed after imidazole addition) at 4 °C. 1.5 mL fractions were collected. Figure 1A illustrates the HisTrap elution profile, Figure 1B illustrates the SDS-PAGE 4-12% gel loaded with 20 µL from fractions A2, A3 and A4 in non-reducing (left hand side) and reducing (right hand side) conditions. SDS-PAGE Protein markers in panel 1D. Fractions A1-A3 were pooled, and 0.5 mL of size-exclusion chromatography buffer (SECB) added: 20 mM HEPES pH 7.5 and 100 mM NaCl. The resulting 5 mL were loaded on a 5 mL loop and injected on a S75 16/60 size exclusion column (GE Healthcare) equilibrated in SECB at 4 °C, injection and run at 0.5 mL/min. Figure 1C shows the S75 16/60 elution profile. Fractions C9➔D4 were pooled and concentrated to a volume

of 1.9 mL, absorbance OD_{280}=7.1. Using a calculated ε_{280}=2.0 $(mg/mL)^{-1}$ cm^{-1} this corresponds to a concentration of 3.5 mg/mL and a total yield of 6.65 mg *Em*PNGAse F.

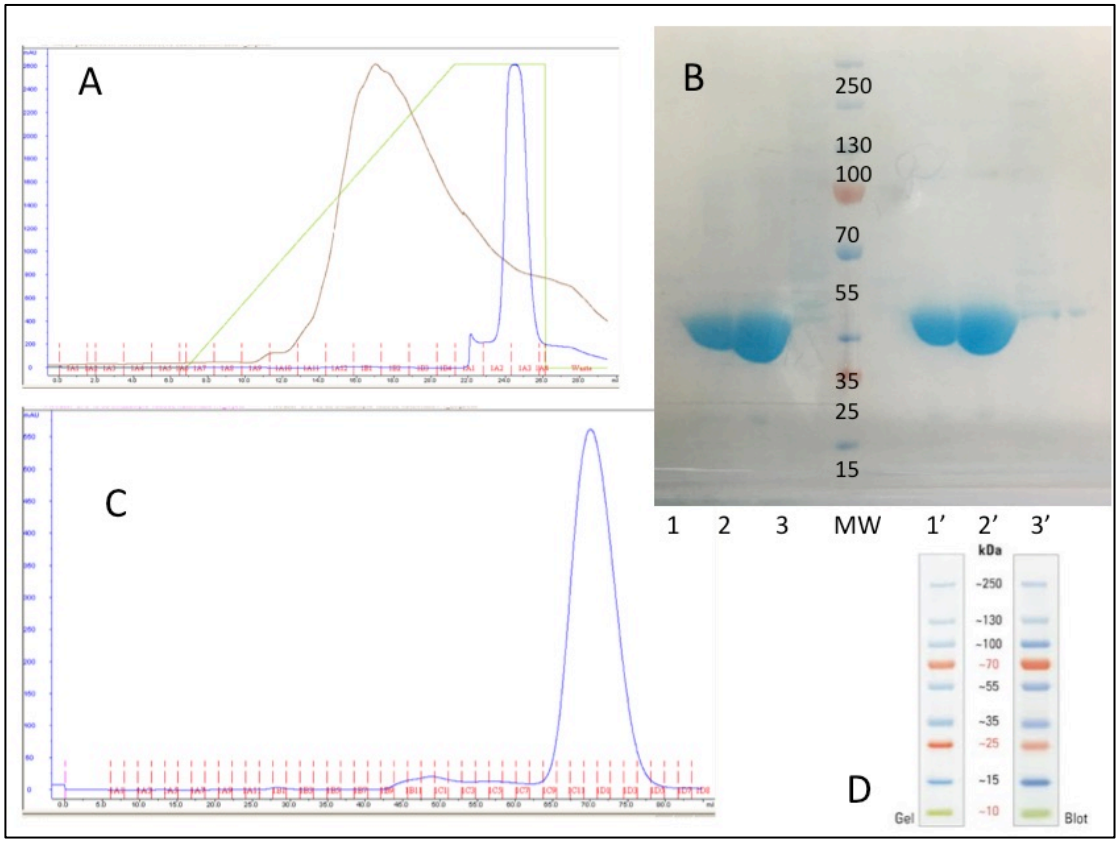

Figure 1. *Em*PNGAse F purification. A: Elution profile from IMAC purification on 1 mL HisTrap column. **Blue: A_{280} (mAU). Green: % elution buffer. Brown: conductivity.** B. SDS-PAGE 4-12% IMAC fractions: 1,1': HisTrap A2 fraction; 2,2': HisTrap A3 fraction; 3,3': HisTrap A4 fraction. Primed lanes contain **50 mM dithiothreitol (**DTT**)** as a reducing agent. All **lanes loaded with:** 20 µL sample plus 4 µL 4X dye. Denatured 1' at 85 °C. Gel run at 200 V constant voltage for 50' in MOPS buffer. C. Elution profile from size exclusion purification **of pooled HisTrap A2-A4 fractions** on S75 16/60 column. D. Protein SDS-PAGE MW markers.

Enzymatic deglycosylation. Five different glycoproteins were tested in as many deglycosylation assays with the batch of *Em*PNGase F purified as described in the previous paragraphs. Glycoprotein's concentration: [*Ct*UGGT:Sep15]=6 mg/mL; [*Hs*Factor H]=44.6 µM; [*Gg*IgY]=86.5 µM; [$CtUGGT_{Kif}$]=6 mg/mL; [$HsTrop2_{EC2}$]=12 mg/mL. Each 20 µL reaction pot contained 3 µL of glycoprotein and 1 µL of *Em*PNGase F at 3.5 mg/mL, plus SECB. Reactions proceeded at 37 °C overnight then were quenched with reducing SDS-loading dye - followed by heat denaturation at 85 for 1'. Figure 2 illustrates the 4-12% gradient SDS-PAGE gel after the deglycosylations: constant voltage 200 V, 50' in MOPS buffer. The *Hs*Factor H and $HsTrop2_{EC2}$ show a shift to lower molecular weights. The proteins contain 9 and 4 putative N-linked glycosylation sites, respectively. The MW shifts are compatible with the putative number of sites and an average complex glycan molecular weight of 2.5 kDa.

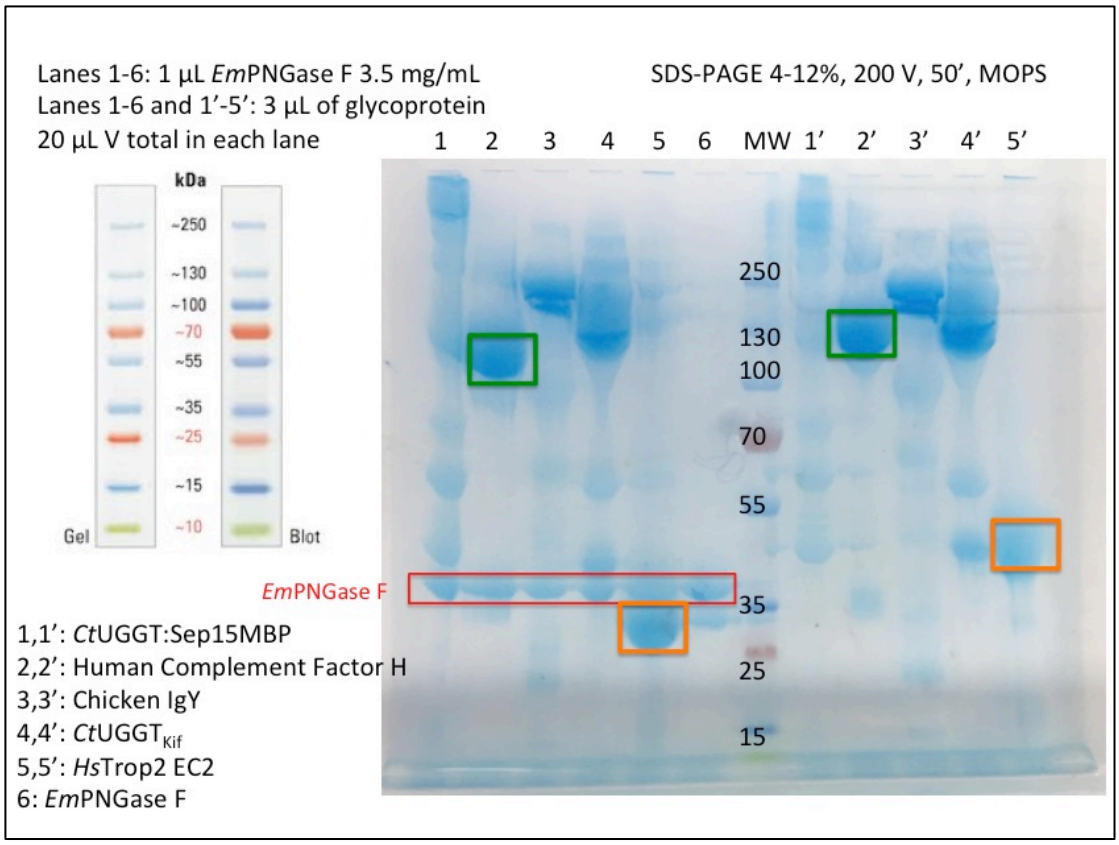

Figure 2. Deglycosylation assays with *Em*PNGAse F. Lanes 1,1': *Ct*UGGT:Sep15; Lanes 2,2': *Hs*Factor H; 3,3': *Gallus gallus* IgY; 4,4': *Ct*UGGT$_{Kif}$; 5,5': *Hs*Trop2$_{EC2}$ (*aka* *Hs*Trop2 extracellular domain double mutant R87A/T88A); lane 6: PNGaseF control. Primed lanes contain no *Em*PNGase F. 4-12% gradient gel run at constant voltage 200 V, 50' in MOPS buffer. The *Hs*Factor H and *Hs*Trop2$_{EC2}$ (boxed in green and orange) show a shift to lower molecular weights. The proteins contain 9 and 4 putative N-linked glycosylation sites, respectively.

Serial dilution of *Em*PNGase F in overnight deglycosylation of *Hs*Trop2-EC2. A serial dilution of *Em*PNGase F was tested for its ability to deglycosylate recombinant *Hs*Trop2$_{EC2}$ (*aka* *Hs*Trop2 extracellular domain double mutant R87A/T88A, 28 kDa nominal MW) at 37 °C overnight. A volume of 9 µL of *Hs*Trop2$_{EC2}$ at 1.2 mg/mL (about 10.8 µg of protein) was mixed with a 1:10 dilution series of a solution of *Em*PNGase F at 0.35 mg/mL solution in SECB. The deglycosylation reactions proceeded at 37 °C overnight and were quenched by addition of reducing SDS-PAGE buffer. Figure 3A illustrates the SDS-PAGE gel analysis of the deglycosylation reactions. Concentrations of *Em*PNGase F of 3.5 $\times 10^{-2}$ mg/mL and 3.5 $\times 10^{-3}$ mg/mL fully deglycosylated the sample (lanes 2,3) as well as the maximum concentration tested (lane 1, 3.5 $\times 10^{-1}$ mg/mL). Three main deglycosylated species are formed overnight (one at 28 KDa, on at 35 kDa and one slightly higher than 35 kDa in mass).

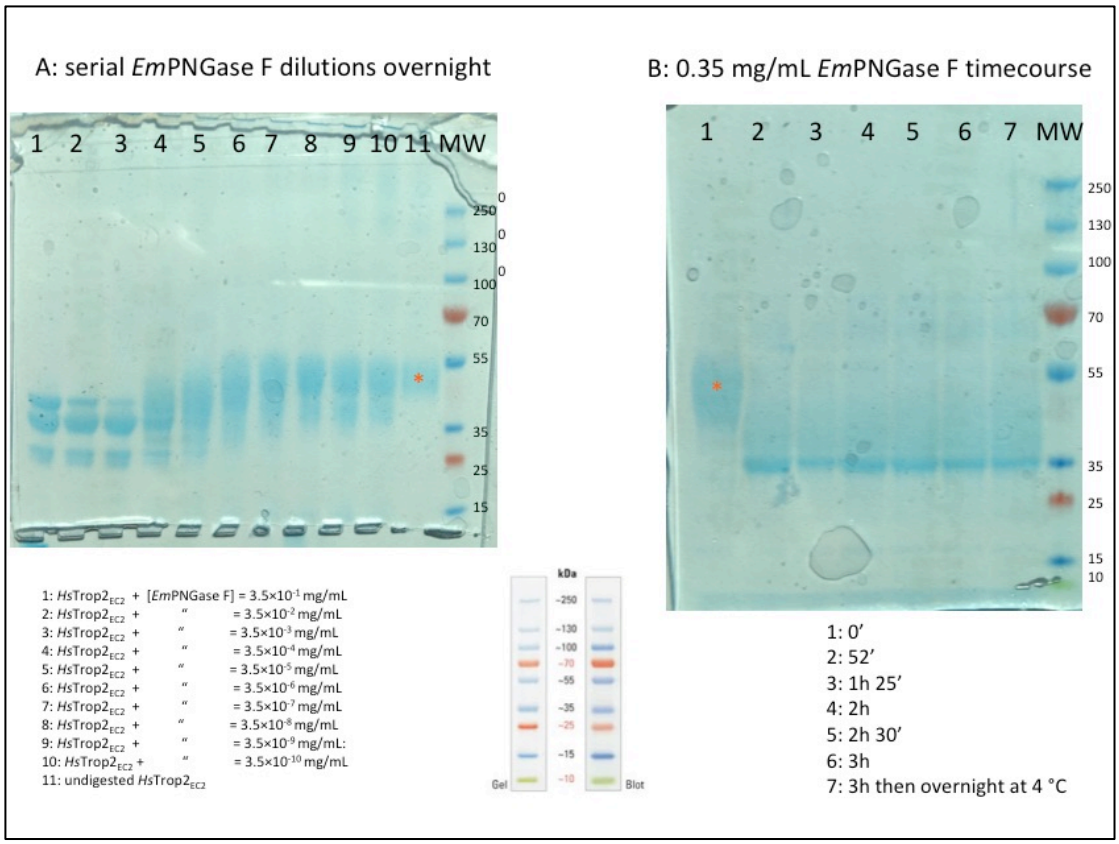

Figure 1 Assay of PNGase F amounts and time course of deglycosylation of *Hs*Trop2-EC2. **A.** SDS-PAGE analysis of deglycosylation of 10.8 µg of *Hs*Trop2-EC2 with a 10-fold dilution series of PNGase F overnight at 37 °C. B. Timecourse of deglycosylation of the same amount of *Hs*Trop2-EC2 with 0.35 mg/mL of PNGase F for three hours at 37 °C.

Time course of *Em*PNGase F deglycosylation of *Hs*Trop2-EC2.

*Em*PNGase F was tested for its ability to deglycosylate recombinant *Hs*Trop2$_{EC2}$ (*aka Hs*Trop2 extracellular domain double mutant R87A/T88A) at 37 °C over the course of three hours. A volume of 9 µL of *Hs*Trop2$_{EC2}$ at 1.2 mg/mL (about 10.8 µg of protein) was mixed with 1 µL of *Em*PNGase F to a final concentration of 0.35 mg/mL solution. The deglycosylation reactions proceeded at 37 °C and were quenched by addition of reducing SDS-PAGE buffer. Figure 3B illustrates the SDS-PAGE gel analysis of the deglycosylation reaction at time points of 50', 1h 30', 2 h, 2h 30' and 3h. After 50' it is apparent the *Hs*Trop2$_{EC2}$ is fully deglycosylated and runs at 35 kDa.