Title:

Transient expression of an anthrax decoy protein in *Nicotiana benthamiana*: Impacts of N-glycosylation on protein expression, stability and function

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Anthrax is a severe infectious disease caused by *Bacillus anthracis*. The spores can be produced easily and released in air as a biological weapon, leading to a fatality rate of 45% even after aggressive treatments. Antitoxins based on receptor-decoy binding show promising advantages over an antibody-based strategy since it is difficult to engineer toxins to escape the inhibitory effect of the decoy without compromising binding to its cellular receptor. The main anthrax receptor on the cell surface is Capillary Morphogenesis Gene-2 (CMG2), which binds to the protective antigen (PA) of anthrax and leads to toxin endocytosis. To improve the stability and circulatory half-life of the decoy protein, we have expressed a Fc-fusion form of the anthrax decoy protein, CMG2-Fc. CMG2-Fc was produced in *Nicotiana benthamiana* plants utilizing *Agrobacteria* infiltration, resulting in expression up to 578 mg/kg of leaf fresh weight. To investigate the effects of N-glycosylation on protein properties, CMG2-Fc variants with three types of N-glycoforms were produced, including plant complex type (APO), a mixture of complex and oligomannose-type (ER) and aglycosylated (Agly). The expression levels of APO and ER variants were 2-fold higher than the Agly variant, suggesting stabilizing effects of N-glycans on CMG2-Fc during *in-planta* production. The toxin neutralization potency of CMG2-Fc variants was tested in a cell-based assay, where the EC_{50} values for APO and Agly variants were lower than for the ER variant. The binding kinetics between CMG2-Fc and PA were determined utilizing biolayer interferometry at room temperature and body temperature (37 ^{o}C). In both cases, the dissociation rate constant (K_D) for all variants were in sub-nano molar level, demonstrating a tight binding regardless of glycosylation. The protein thermostability was examined using an ELISA, where the fraction of functional ER variant decayed significantly after overnight incubation at 37^{o}C. This agrees with the higher EC_{50} observed in TNA for the ER variant. In parallel, molecular dynamics (MD) simulations were performed to understand experimental observation at the molecular level. In MD simulation, mannose-type glycoform (MAN8) exhibits a higher hydrophobic solvent accessible surface areas (SASA), indicate a potential higher aggregation tendency of the ER variant. This work combines experimental and simulation approaches to study the impacts of N-glycosylation on protein properties, which potentially serves as a guideline for optimizing biologics design and expression in plants.