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Stability and pre-formulation development of a plant-produced anthrax vaccine candidate

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ABSTRACT

Second generation anthrax vaccines focus on the use of recombinant protective antigen (rPA) to elicit a strong, toxin neutralizing antibody responses in immunized subjects. The main difference between the rPA vaccines compared to the current licensed vaccine, anthrax vaccine absorbed (AVA), is the rPA vaccines are highly purified preparations of only rPA. These second generation rPA vaccines strive to elicit strong immune responses with substantially fewer doses than AVA while provoking less side effects. Many of the rPA candidates have shown to be effective in pre-clinical studies, but most of the second generation molecules have stability issues which reduce their efficacy over time. These stability issues are evident even under refrigerated conditions and thus emphasis has been directed to stabilizing the rPA molecule and determining an optimized final formulation. Stabilization of vaccines for long-term storage is a major challenge in the product development life cycle. The effort required to identify suitable formulations can be slow and expensive. The ideal storage for stockpiled vaccines would allow the candidate to withstand years of storage at ambient temperatures. The Fraunhofer Center for Molecular Biotechnology is developing a plant-produced rPA vaccine candidate that shows instability when stored under refrigerated conditions in a solution, as is typical for rPA vaccines. Increased stability of our plant-produced rPA vaccine candidate was achieved in a spray dried powder formulation that could eliminate the need for conventional cold chain allowing greater confidence to stockpile vaccine for civilian and military biodefense.

1. Introduction

The majority of second generation anthrax vaccines, for active immunization against *Bacillus anthracis*, use the immunogen protective antigen (PA). Vaccinations with native or recombinant PA (rPA) generate high toxin neutralization titers and offer protective immunity. In pre-clinical efficacy studies rPA has been used with various adjuvants, but mostly with aluminium salts [1,2]. The Alhydrogel rPA formulation is the only FDA-approved anthrax vaccine (Anthrax Vaccine Adsorbed [AVA]) [3]. AVA is produced from filtrates of a *B. anthracis* culture and consists of PA as the primary immunogen.

PA is non-toxic, but associates with either lethal factor (LF) or edema factor (EF) to form lethal toxin (LT) or edema toxin (ET), respectively. Upon recognition and binding to cell surface receptors, PA is cleaved by furin and multimerizes to form a membrane spanning pore [4]. The pore allows transport of LF or EF into the cell cytosol where their enzymatic activity leads to cytotoxicity [5]. Vaccination with PA or rPA generates antibodies that bind PA, blocking its interaction with cell receptors and/or EF/LF leading to toxin neutralizing antibody activity (TNA). PA based vaccines are well tolerated and effective, but PA suffers from inherent instability. Known proteolytic sensitive sites and several chemically instable arginines decrease the proteins overall stability [6–8]. Even with these liabilities, PA vaccines are being stockpiled and are considered one of the best measures of protection in the event of an anthrax terrorist attack.

Work to stabilize rPA, includes point mutations to remove the furin cleavage site and limit the protein instability during purification [9], expression as an oral vaccine to eliminate downstream processing [10], and formulation of purified PA into non-liquid

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formulations. Dried formulations have been prepared for cutaneous micro-needle and intranasal delivery [11] and shown to have stability at 25 °C or 40 °C for up to a month [12]. Other dried formulations remained immunogenic after 2.5 years of room temperature storage [13]. The results demonstrate that there is a precedent for stabilizing PA with drying technologies.

Herein we report on spray dried formulation development of a plant-produced, deglycosylated rPA vaccine candidate (pp-dPA83). Spray drying has been used for over half a century in preparation of pharmaceutical excipients, is well controlled and scalable, and is an alternative to lyophilization for the production of dry therapeutics [14]. The continuous spray drying process applied to pp-dPA83 minimizes sheer on the antigen, rapidly evaporates the aqueous carrier and generates a stable powder. The pp-dPA83 vaccine candidate was produced in a transient plant-based expression system, co-expressed with the deglycosylation enzyme peptide-N-glycosidase F (PNGase F) and purified from *Nicotiana benthamiana* [15]. Plants are a good alternative for recombinant protein production as they are cost-effective, offer high target expression and do not harbour mammalian pathogens [16–19] which is important for manufacturing of vaccines and therapeutics. Furthermore, plants are able to perform post-translational modifications and complex folding similar to native protein expression [20].

2. Materials and methods

2.1. Protein

pp-dPA83 was produced in *N. benthamiana*, similarly as described in Ref. [15]. Briefly, nucleotides 30–764 of *B. anthracis* PA (Genebank AN: P13423) were optimized for expression in *N. tabacum* and cloned into the binary vector pGR-D-PA83-1-PNGaseF including an N-terminal pathogenesis-related protein 1a single peptide. A C-terminal hexa-histidine (6xHis) purification tag and a KDEL endoplasmic reticulum retention signal were added. Transformed *Agrobacterium tumefaciens* were vacuum infiltrated into *N. benthamiana* and harvested after four days. Aerial biomass was homogenized and clarification was achieved with centrifugation and filtration. pp-dPA83 was purified by immobilized metal affinity, ion-exchange, and size exclusion chromatography (SEC). The resulting protein was >90% pure as determined by SDS-PAGE, reverse phase chromatography and analytical SEC. pp-dPA83 was stored in 1x phosphate buffered saline (PBS), pH 7.4 at −70 °C. pp-dPA83 stored at −70 °C was used as reference sample throughout these studies.

2.2. Spray drying

Formulations of the vaccine candidate were prepared in aqueous media at 10 wt% total solids. Spray drying was performed on a custom built, laboratory-scale dryer (BLD-35) (Bend Research, Bend, Oregon) equipped with a two-fluid atomizer nozzle set to 8 psi. Drying gas (nitrogen) flow rate was 30 kg/h with a liquid solution feed rate of 4 mL/min. Inlet temperature ranged from 100 to 120 °C, with a set outlet temperature of 58 °C. Powder was captured following drying with a custom cyclone. Powder aliquoting was executed under <10% relative humidity (RH) and stored in sealed Aluminium-Mylar bags at ambient temperature including a desiccant packet (Sorbant Systems, Los Angeles, CA).

2.3. Dry powder reconstitution

Reconstitution was achieved by addition of 2 mL of Milli Q water (18 Ω, 0.2 μm filtered) to 0.2 g of powder. The wetted powder was vortexed for 10 s followed by a 2-min rest and a second vortex.

2.4. Differential scanning calorimetry (DSC)

Two to 10 mg samples were loaded as loose powder into non-hermetic Instrument Specialists (Twin Lakes, Wisconsin) DSC pans and examined using a TA Instruments (New Castle, Delaware) DSC1000 to measure the glass transition temperature (Tg). The typical scan range was −40.00 °C to 220.00 °C. Program settings also included modulation of ±1.50 °C every 60 s with a ramp of 2.50 °C/min with a dry nitrogen purge (50 mL/min).

2.5. Karl Fischer (KF) assay

Triplicate samples containing 20–60 mg of powder were added to a vial and closed under controlled conditions (<10% RH). Vials were loaded onto a coulometric KF oven with a Metrohm 774 oven sample processor and Metrohm 851 Titrando (Metrohm, Riverview, Florida). Samples were heated to 150 °C and the headspace was removed for analysis with a drift setting of 15 μg/min. System suitability was tested to confirm that the standard (Fluka, Hydranol 34693 Water Std KF-Oven) was within 1% of label claim (theoretical 5.2 wt%). Data was evaluated by Tiamo 2.2 analysis (Metrohm control and database software).

2.6. Scanning electron microscopy (SEM)

A Hitachi S-3400 N was used to image dry powders. Samples were tapped onto a glue tab (Ted Pella Inc. Redding, California) on an aluminium specimen mount (Ted Pella No. 16111). The post was tapped several times to confirm attachment and then placed in a Anatech Hummer 6.2 sputter coater system (Anatech Limited, Battle Creek, Michigan) and coated with Au/Pd for 8 min at approximately 15 mA to 20 mA. Samples were placed in the SEM running at 30 kV in variable-pressure mode.

2.7. Powder X-ray diffraction (PXRD)

Samples were examined using a Bruker AXS D8 Advance diffractometer (Bruker Corporation, Germany) with positional autosampler arm. Samples (∼30–100 mg) were packed in 0.5-mm-deep zero-background-holder sample cups and spun in the j plane at 30 rpm to minimize crystal orientation effects. The X-ray source – CuKα (λ = 1.54 Å) – was operated at 45 kV and 40 mA. Data for each sample were collected from 4° to 40° on the 2Θ scale over 30 min in a continuous-detector scan mode at a scan speed of 2 s/step and a step size of 0.04°/step.

2.8. SDS-PAGE

Reconstituted, spray dried pp-dPA83 and a reference sample were prepared in reducing 5× sample buffer and heated for 1 min (95 °C). Samples were electrophoresed on a 10% TGX™ SDS-PAGE (BioRad) gel run at 20 mA/gel with a maximum of 200v. Gels were stained with Gel Code Blue Stain Reagent (Thermo) and after destaining, scanned with a HP flatbed scanner.

2.9. Isoelectric focusing (IEF)

IEF PAGE was performed with pH 3.0–7.0 IEF gels using the manufacturer's recommended anode and cathode buffers (Invitrogen). IEF markers were obtained from Thermo and covered pI range 3–10 (SERVA). Fixed gels were stained with colloidal Blue Staining kit (Invitrogen) and after destaining, gels were scanned with a HP flatbed scanner.

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