

Expression, purification, and characterization of the *Necator americanus* aspartic protease-1 (*Na*-APR-1 (M74)) antigen, a component of the bivalent human hookworm vaccine

Christopher A Seid^{1,2,#}, Elena Curti^{1,2,#}, R Mark Jones^{3,#}, Elissa Hudspeth^{1,2}, Wanderson Rezende^{1,2}, Jeroen Pollet^{1,2}, Lori Center^{1,2}, Leroy Versteeg^{1,2}, Sonya Pritchard³, Konstantin Musiychuk³, Vidadi Yusibov³, Peter J Hotez^{1,2,4}, and Maria Elena Bottazzi^{1,2,4,*}

¹Departments of Pediatrics and Molecular Virology and Microbiology; National School of Tropical Medicine; Baylor College of Medicine; Houston, TX USA; ²Sabin Vaccine Institute and Texas Children's Hospital Center for Vaccine Development; Houston, TX USA; ³Fraunhofer Center for Molecular Biotechnology; Newark, DE USA; ⁴Department of Biology; Baylor University; Waco, TX USA

[#]These authors equally contributed to this work.

Keywords: product development partnership, vaccine, human hookworm, process development, tobacco plant expression, aspartic protease

Over 400 million people living in the world's poorest developing nations are infected with hookworms, mostly of the genus *Necator americanus*. A bivalent human hookworm vaccine composed of the *Necator americanus* Glutathione S-Transferase-1 (*Na*-GST-1) and the *Necator americanus* Aspartic Protease-1 (*Na*-APR-1 (M74)) is currently under development by the Sabin Vaccine Institute Product Development Partnership (Sabin PDP). Both monovalent vaccines are currently in Phase 1 trials. Both *Na*-GST-1 and *Na*-APR-1 antigens are expressed as recombinant proteins. While *Na*-GST-1 was found to express with high yields in *Pichia pastoris*, the level of expression of *Na*-APR-1 in this host was too low to be suitable for a manufacturing process. When the tobacco plant *Nicotiana benthamiana* was evaluated as an expression system, acceptable levels of solubility, yield, and stability were attained. Observed expression levels of *Na*-APR-1 (M74) using this system are ~300 mg/kg. Here we describe the achievements and obstacles encountered during process development as well as characterization and stability of the purified *Na*-APR-1 (M74) protein and formulated vaccine. The expression, purification and analysis of purified *Na*-APR-1 (M74) protein obtained from representative 5 kg reproducibility runs performed to qualify the *Na*-APR-1 (M74) production process is also presented. This process has been successfully transferred to a pilot plant and a 50 kg scale manufacturing campaign under current Good Manufacturing Practice (cGMP) has been performed. The 50 kg run has provided a sufficient amount of protein to support the ongoing hookworm vaccine development program of the Sabin PDP.

Introduction

New estimates from the Global Burden of Disease Study 2010 (GBD 2010) indicate that 439 million people residing in impoverished areas of South and Southeast Asia, Oceania, Africa, and Latin America live with hookworms in their gastrointestinal tracts.^{1,2} The most prevalent human hookworm species is *Necator americanus*, which has the ability to cause intestinal blood loss leading to anemia.³ GBD 2010 has further determined that hookworms are now a leading cause of anemia in many developing countries,⁴ especially affecting children⁵ and pregnant women.⁶

Adult hookworms cause anemia by ingesting human blood at their site of attachment in the small intestine. In order for the parasite to utilize blood as a source of nutrition, its gut is lined with proteases which catalyze the hydrolysis of host hemoglobin.^{7,8} A major enzyme involved in the process of hemoglobin hydrolysis is *N. americanus* Aspartic Protease-1 (*Na*-APR-1), a 45 kDa protein member of the cathepsin D aspartic protease family. The *Na*-APR-1 protein is expressed in the gut of the adult *N. americanus* parasites where it functions in concert with the cysteine protease *Na*-CP-3 and the metalloprotease *Na*-MEP-1 to digest the hemoglobin tetramer.^{7–9} Due to its role

© Christopher A Seid, Elena Curti, R Mark Jones, Elissa Hudspeth, Wanderson Rezende, Jeroen Pollet, Lori Center, Leroy Versteeg, Sonya Pritchard, Konstantin Musiychuk, Vidadi Yusibov, Peter J Hotez, and Maria Elena Bottazzi

*Correspondence to: Maria Elena Bottazzi; Email: bottazzi@bcm.edu

Submitted: 12/17/2014; Revised: 03/13/2015; Accepted: 03/27/2015

<http://dx.doi.org/10.1080/21645515.2015.1036207>

This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

in hemoglobin digestion, *Na*-APR-1 was selected as a candidate for the development of a hookworm vaccine since neutralization of this crucial enzyme would result in starvation of the parasite and concomitantly could lead to parasite death and the prevention of blood loss in the infected host. In support of this hypothesis, vaccination of dogs with the canine homolog of the wild type *Na*-APR-1 (*Ancylostoma caninum*-APR-1) resulted in the production of neutralizing antibodies against the hookworm hemoglobinase and thus, higher level of hemoglobin in the blood of vaccinated dogs compared to the non-vaccinated control group, as well as a reduction in both egg counts and adult parasite burden in the gut of the infected host.¹⁰ Likewise, hamsters immunized with *Ac*-APR-1 also showed a significant reduction in worm burden.¹¹ These findings led to the development of an experimental human hookworm vaccine comprised of *Na*-APR-1, together with *N. americanus* glutathione S-transferase 1 (*Na*-GST-1) a unique hookworm enzyme that functions in heme binding and detoxification.¹²⁻¹⁵ The goal of the vaccine strategy is to elicit neutralizing antibodies to both enzymes which would inhibit the parasites ability to detoxify and eliminate heme as well as utilize host hemoglobin for survival.

During development of the aspartic protease based component of the human hookworm vaccine, the catalytic active site of *Na*-APR-1 was inactivated by mutating the aspartic acid residues at positions 113 and 300 to alanine residues (*Na*-APR-1 (M74)). The rationale for the change was to circumvent potential toxicities of immunizing humans with an active proteolytic enzyme. Vaccination of canines with *Na*-APR-1 (M74) followed by a heterologous challenge with the canine hookworm *Ancylostoma caninum* resulted in a significant reduction in both parasite egg burden and weight loss.⁹ In addition, vaccination with *Na*-APR-1 (M74) induced antibodies that not only bound to the native enzyme in the parasite gut, but also neutralized enzymatic activity of both wild type *Na*-APR-1 as well as APR-1 orthologues from 3 other hookworm species that infect humans.⁹

The production of *Na*-APR-1 (M74) protein at high yields and with adequate stability, however, has been challenging due to the very low expression levels observed in *P. pastoris* and the lack of solubility of the *Escherichia coli* expressed protein. Attempts to solubilize *Na*-APR-1 (M74) with chaotropic agents followed by refolding the protein proved difficult to achieve. In contrast to *P. pastoris* and *E. coli*, evaluation of the tobacco plant *N. benthamiana* as a potential expression system resulted in both acceptable yields and stability of the aspartic protease. In support of using this expression system for process development and ultimately scale up, plants have been shown to be a good alternative for recombinant protein production due to their ability to generate a high biomass with the potential for high protein expression.¹⁶ Being a eukaryotic system, plants are able to perform post-translational modifications to the target protein, thus making the protein more similar to the native protein. Furthermore for the purpose of vaccine manufacturing and safety concerns, plants do not harbor pathogens which can endanger humans.¹⁷⁻¹⁹ The cost of producing a recombinant protein in transgenic plants as compared to *E. coli* or other expression hosts could be 10–50 fold lower depending on the

plant strain used.²⁰ There are many examples of recombinant proteins manufactured in plants which are being used as effective vaccines for diseases such as influenza, anthrax, and plague.^{21,22,24,28} However, heterologous protein expression in plants does face many hurdles that must be overcome. Recombinant proteins which express at low levels in plants require considerable effort to isolate from the milieu of total plant proteins. Recovery can be compromised when isolating a low abundance target as described herein. While no products transiently expressed in tobacco have yet been approved by the FDA, Taliglucerase alfa which is produced in transgenic carrot cells at commercial scale by Protalix BioTherapeutics was approved in 2012. Facilities which utilize controlled plant growth and infiltration processes for heterologous protein expression in whole plants that conform to current Good Manufacturing Practice (cGMP) guidelines have been described.^{32,33} Although our system is using a non-transgenic plant which does not require the permits and strict containment plans required for transgenic plants,³¹ the system has yet to be tested at commercial manufacturing scale. With plant expression technology, there are additional hurdles which must be overcome including issues related to safety of potential contaminating proteins, cultivation, and downstream processing.³⁴

Fraunhofer USA Center for Molecular Biotechnology (FhCMB; Newark, DE) has developed a transient plant-based hybrid expression system for the production of vaccine antigens by infiltration of *N. benthamiana* with *Agrobacterium tumefaciens* which harbor an expression vector containing viral vector sequences for controlling target gene expression. The FhCMB system has been successfully implemented and utilized to produce Phase I material for 5 human clinical trials. *Na*-APR-1 (M74) without the native pro-sequence (residues 1–73) was codon optimized for expression in *N. benthamiana* and engineered to include a PR-1a signal sequence at the N-terminus for protein secretion and a C-terminal 6-HIS tag to ease purification. *Na*-APR-1 (M74) was then cloned into the hybrid vector system and used to express *Na*-APR-1 (M74) at ~300 mg protein per kg plant biomass with approximately 60% solubility. Purification of the target protein resulted in final yields of 7–10 mg per kg biomass with a purity of >90%. Here, we describe the process development and characterization strategy of the in process and purified protein samples obtained from representative 5kg scale reproducibility runs performed to qualify the *Na*-APR-1 (M74) production process. In addition, we present data from the stability assessment of *Na*-APR-1 (M74) purified at the 5Kg scale. The process reported here was successfully transferred to a pilot plant and a 50 kg scale manufacture has been performed under current Good Manufacturing Practice in support of Phase I trials.

Results

Expression and purification of *Na*-APR-1 (M74) from *N. benthamiana*

The general structure of the *Na*-APR-1 (M74) expression cassette and the amino acid sequence is shown in Figures 1A–B,

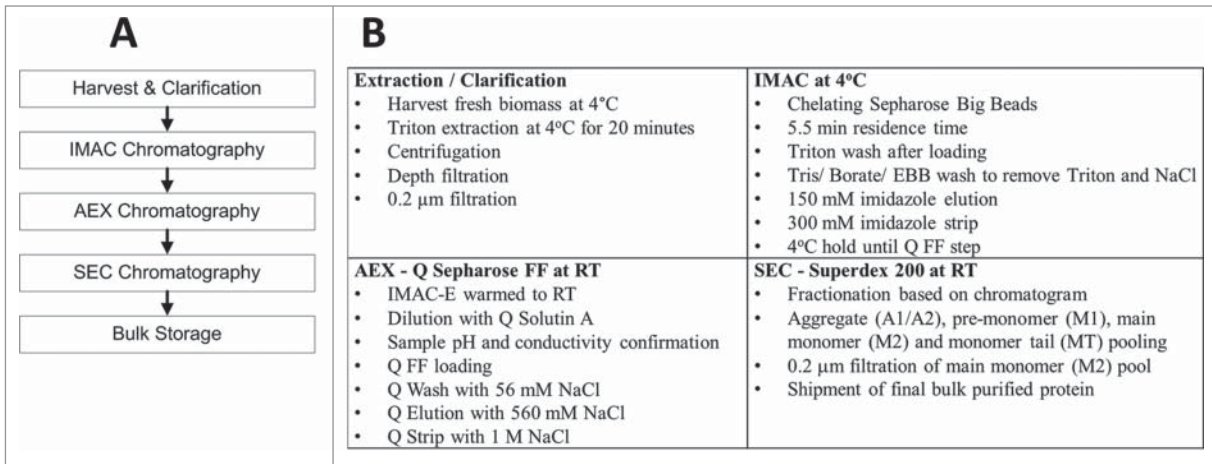


Figure 2. Process Schematic Overview. (A) Schematic of the purification process of *Na*-APR-1 (M74). (B) Summary of the process steps for the harvest/clarification and for each chromatography step.

indicated a QFF yield of approximately 17 ± 4 mg *Na*-APR-1 (M74) per kg biomass as determined by densitometric comparison to BSA standards of known concentrations (data not shown). Based on the quantitative SDS-PAGE analysis, the Q pool contained 91 ± 15 mg of *Na*-APR-1 (M74) (average from 3 5kg preparations). Prior to performing SEC chromatography, the Q pool was divided into equal fractions in order to keep the SEC load volume between 3.4 – 4.0% of the CV for each of the SEC runs to ensure sufficient resolution and separation of proteins. Fraction collection during each SEC run was based on 1) separation of the *Na*-APR-1 (M74) monomer from the aggregate

contaminants and 2) a cumulative concentration of not less than 0.2 mg/mL to provide a final acceptable concentration for later formulation. The SEC fractions observed consisted of an aggregate upslope (A1), aggregate downslope (A2), pre-monomer (M1), main monomer (M2) and post monomer tail (MT) portions (Fig. 4A). Separation of the monomer from aggregate was determined by non-reducing SDS-PAGE analysis of the first SEC run fractions (Fig. 4B). Division points were determined by examining each fraction for the presence of high molecular weight contaminants that could be seen in the aggregate downslope (A2) and pre-monomer (M1) fractions (Fig. 4B; lanes 2

(A2) versus lanes 3–8 (M1)). Fractions containing high molecular weight contaminants were not used for SEC pooling. The endpoint for the SEC M2 fraction (main monomer) was based on maintaining the cumulative pooled concentration above 0.2 mg/mL. Reduced SDS-PAGE analysis of the final purified *Na*-APR-1 (M74) protein is shown in Figure 5. Analytical SEC of the IMAC elution pool (IMAC), the Q elution pool (AEX), and the final purified *Na*-APR-1 (M74) protein (Final) is shown in Figure 6. Data indicates that the SEC step significantly reduces the aggregate peak resulting in enrichment of the *Na*-APR-1 (M74) target monomer. The final yield of purified bulk *Na*-APR-1 (M74) after the SEC step was 8.6 ± 2.0 mg per kg biomass based on the average of 3 runs.

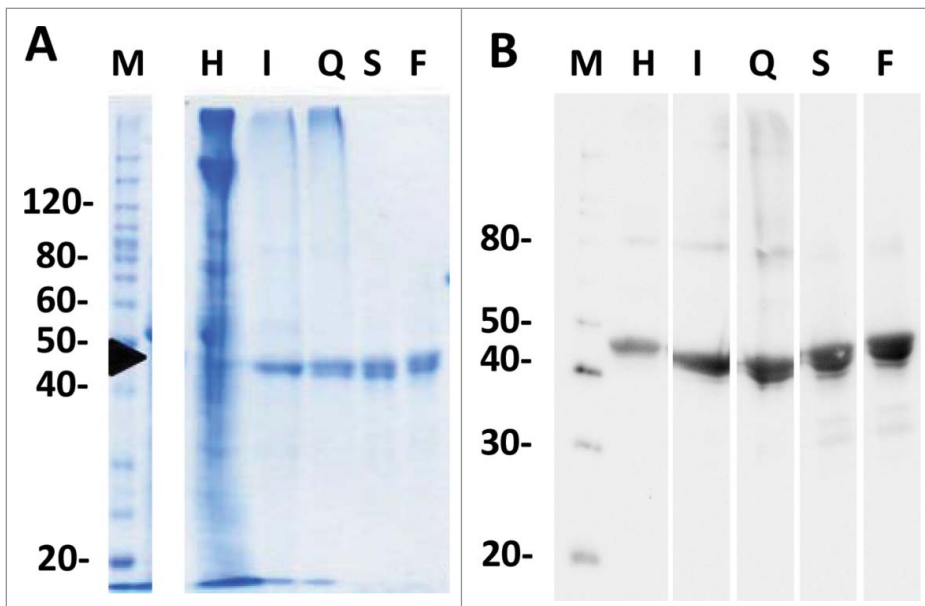


Figure 3. SDS-PAGE analysis of in process samples (A) Coomassie stained SDS-PAGE (10%) of non-reduced purification in process samples; M – molecular weight markers, H – homogenate, I – immobilized metal affinity chromatography (IMAC) eluate, Q – ion exchange chromatography eluent, S – size exclusion chromatography pooled eluent, F – final purified *Na*-APR-1 (M74). (B) Western blot of non-reduced process samples probed with an anti-APR1 primary antibody.

Characterization of the *Na*-APR-1 (M74) and accelerated stability studies
The recombinant *Na*-APR-1 (M74) was characterized after purification to

ensure purity and integrity of the sample prior to vaccine formulation. Characterization included visual inspection, determination of the pH, measurement of the protein concentration by absorbance at 280 nm (Table 2), evaluation of the separation profile by SEC-HPLC, SDS-PAGE followed by Coomassie Blue staining, Silver Staining and Western blot (Fig. 7). Immediately after elution, the protein sample was analyzed and aliquots were placed at 2–8°C, 25° or 37°C for a time period of 30 days for accelerated stability studies. At days 7, 15 and 30, samples were removed from the storage temperatures and tested for the same parameters as described above. Although the pH and protein concentration of these samples did not vary significantly across the storage conditions tested, degradation products were detected by SDS-PAGE followed by Coomassie Blue staining and Silver Staining after 7 days (Fig. 8A,B), and increasingly after 15 (Fig. 8D,E) and 30 days (Fig. 8G,H). Similar degradation products were observed when the samples were analyzed by Western blot after 7 days, 15 days, and 30 days (Fig. 8C,F,I). The SEC-HPLC elution profiles for the different time points are shown in Figures 9A–I.

Protein stability after freeze/thaw (F/T)

The study was carried out as described in Curti et al., 2014. After each freeze/thaw cycle, *Na*-APR-1 (M74) was characterized by the analytical methods previously described. The data indicated a progressive, but subtle degradation of the molecule after each freeze-thaw cycle. The samples maintained their color and appearance properties as well as pH, but a subtle increase of the absorbance reading was observed upon the last freeze/thaw cycle (Table 2B). When analyzed by SDS-PAGE, only traces of *Na*-APR-1 (M74) degradation products were observed at each freeze thaw cycle (Fig. 10D) which were detected by Western blot (Fig. 10E) and SEC-HPLC (Fig. 10A–C). While the samples still retained an SEC-HPLC elution time of approximately 13 minutes, the overall SEC-HPLC elution profiles of the samples after the second and third freeze/thaw cycles indicated increasing signs of degradation, with a reduction in the sample peak area ($\mu\text{V}\cdot\text{sec}$) and peak height (μV) (Table 2C). The performance of *Na*-APR-1 (M74) upon freeze/thaw was assessed across multiple lots and the data gathered were comparable (data not shown).

Long term stability testing of *Na*-APR-1 (M74) protein

A real time stability study was also performed on *Na*-APR-1 (M74) and data have been collected for up to 24 months from the date of purification. Samples were removed from the –80°C freezer and characterized at specified time points. For all time points tested, the samples maintained the color and appearance properties as colorless homogenous solutions free of particles (data not shown). In addition, the pH as well as the concentration of the protein solution did not vary significantly

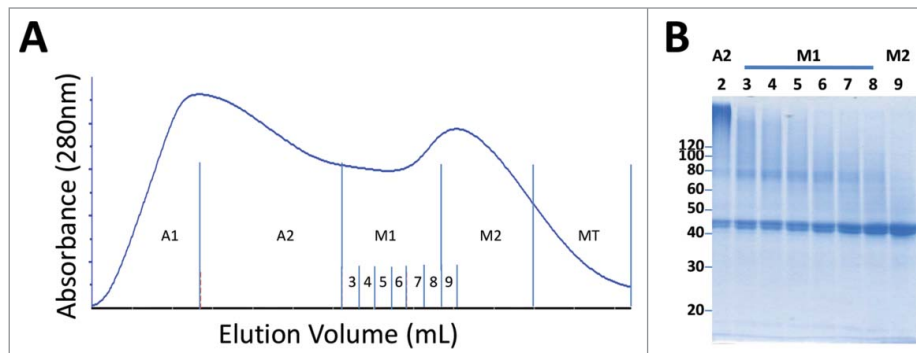


Figure 4. Representative elution profile for preparative SEC. Fractions include aggregate upslope (A1), aggregate downslope (A2), pre-monomer (M1), main monomer (M2) and monomer tail (MT). (B). SDS-PAGE (non-reducing, denatured) analysis of selected SEC fractions including A2, M1 with sub-fractions, and the leading edge of M2. The clearance of high molecular species eluting in A2 and M1 fractions was the impetus for limiting M2 as the main product peak.

between time points tested demonstrating that the protein retained its release specifications and characteristics at manufacture for the entire time of the study when stored at –80°C. When analyzed by SDS-PAGE and by SEC-HPLC, degradation of *Na*-APR-1 (M74) was not observed which confirmed that *Na*-APR-1 (M74) could be stored for a period of time up to 24 months at –80°C (Fig. 11).

Long-term stability of the *Na*-APR-1 (M74) vaccine

The long-term plan for the Human Hookworm vaccine is to co-formulate *Na*-APR-1(M74) with a second hookworm antigen (*Na*-GST-1) which is currently in Phase 1 studies. Both antigens are intended to be formulated with Alhydrogel® adjuvant. The isoelectric point (pI) of *Na*-APR-1 (M74) is 5.26 and the pH of the Imidazole buffer used for formulation is 7.4. Therefore, to

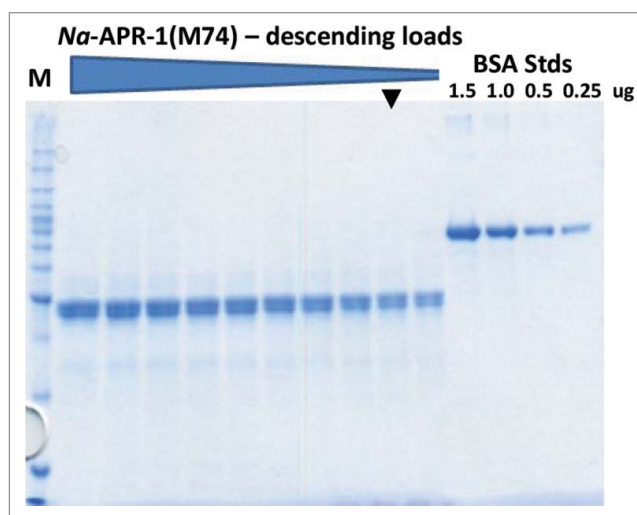


Figure 5. Analysis of the final purified *Na*-APR-1 (M74) protein on a 10% SDS-PAGE gel. Arrowhead indicates a 1.0 μg load. All samples were diluted with 1/5th volume 5x SDS loading dye and loaded in descending volumes from 15 μL to 6 μL in 1 μL increments.

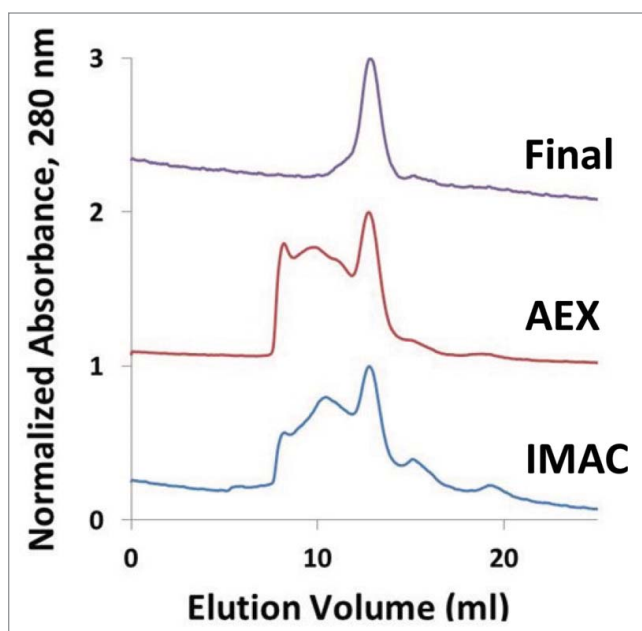


Figure 6. Analytical Fast Pressure Liquid Chromatography-SEC of the IMAC eluent, the anion exchange (AEX) eluent, and the final purified *Na*-APR-1 (M74) protein.

maximize the electrostatic interaction between the aluminum salt and the protein, aluminum hydroxide (Alhydrogel[®]) was chosen as the most suitable adjuvant. In addition, Alhydrogel[®] is already considered safe and approved for human use by the regulatory agencies. To validate the choice of the adjuvant, long-term binding and stability of *Na*-APR-1 (M74) to Alhydrogel[®] was examined. Following storage of the formulated *Na*-APR-1 (M74)/Alhydrogel[®] vaccine at 2–8°C, its stability was investigated by assessing the color and appearance, pH, protein content by the O-phthalaldehyde (OPA) assay, percent of protein bound to Alhydrogel[®], and the identity by SDS-PAGE after desorption of the protein followed by Coomassie Blue staining (data not shown). Samples were withdrawn and tested at specific time points up to a maximum of 24 months. While the color, appearance, and pH were evaluated as previously described, the OPA assay was used to quantify the amount of protein bound to the Alhydrogel[®] surface by separating the surface bound protein fraction of the formulation from the unbound fraction in the supernatant by centrifugation. As shown in Figure 12, when the *Na*-APR-1 (M74)/Alhydrogel[®] formulation was centrifuged to separate the pellet fraction from the supernatant, the *Na*-APR-1 (M74) protein segregated in the pellet fraction and only a very faint band was visible in the supernatant fraction, indicating a tight binding of the molecule to the surface of the Alhydrogel[®].

Discussion

Na-APR-1, the *N. americanus* Aspartic Protease-1, is being developed together with *Na*-GST-1, *N. americanus* Glutathione S-Transferase-1, as lead candidate antigens in a bivalent human

hookworm vaccine by the Sabin Vaccine Institute Product Development Partnership (Sabin PDP). Establishment of an expression system as well as a robust purification process for *Na*-APR-1 (M74) proved, however, to be more difficult than for *Na*-GST-1 (produced in *P. pastoris*).

After screening a number of expression systems including *P. pastoris* and *E. coli* for their ability to produce *Na*-APR-1 (M74), *N. benthamiana* was ultimately selected as the expression host based on the potential scalability of the system and its ability to provide adequate amounts of pure soluble protein to support early clinical development. Even with the successful implementation of this system into the *Na*-APR-1 (M74) development program, some aggregation of the target protein as well as lower than expected yields, persisted. The final selected purification process developed for *Na*-APR-1 (M74) included an extraction and clarification step followed by several chromatographic steps including an immobilized metal affinity (IMAC) step, an ion exchange (QFF) step, and finally a size exclusion (SEC) polishing step.

Mass analysis was performed throughout process development not only to identify the sources and key steps of major protein loss, but also to evaluate the benefit of changing parameters within each step in order to improve product recovery and quality. During the early phase of the development process, the extraction as well as the IMAC purification processes resulted in a significant loss of the target product. This obstacle was partially resolved by changing the extraction buffer from phosphate to Tris allowing for direct loading of the IMAC eluate onto the AEX resin without addition of an ultrafiltration/diafiltration step. In addition, the pH of the IMAC buffers was increased from 7.5 to 8.0 to allow for the AEX step to be performed at room temperature rather than 4°C, which is more suitable for later scale up. For the AEX step, both strong (QFF) and weak (DEAE) anion exchangers were screened for binding efficiency and the QFF matrix was selected based on its greater *Na*-APR-1 (M74) binding capacity compared to the DEAE matrix, at all pH conditions screened.

Despite these optimizations, the overall process recovery remained surprisingly low, with approximately 80% of the IMAC elution being lost through the following QFF step. Further evaluation of the process revealed that the product loss observed at the QFF step was due in part to some product loss during the wash step, but primarily due to the collection of a narrow portion of the relatively broad QFF elution peak. This minimal collection window, however, was necessary in order to 1) maintain the target concentration at ≥ 1.8 mg/mL in order to minimize the SEC load volume and/or the number of SEC cycles and 2) to ensure that the *Na*-APR-1 (M74) eluted from the SEC at a concentration sufficient for downstream formulation (>0.2 mg/ml). In addition to the loss of target protein from the QFF step, a substantial percentage of *Na*-APR-1 (M74) in the QFF eluate was detected as high molecular weight protein suggesting the presence of aggregates (Fig. 6) which supports the narrow fraction window collected.

As a means to reduce aggregation, a number of detergents including anionic, cationic and several zwitterionic detergents were

Table 2: A: Color and appearance, pH measurement and protein content by absorbance at 280nm of *Na*-APR-1 (M74) samples at day 0 and hold at 2–8°C, 25°C, and 37°C for 7, 15 and 30 days; B: Color and appearance, pH measurement and protein content by absorbance at 280nm of *Na*-APR-1 (M74) samples subjected to the 3 freeze/thaw cycles; C: SEC HPLC elution parameters of *Na*-APR-1 (M74) subjected to the 3 freeze/thaw cycles

Sample	Parameter			C: SEC HPLC Elution parameters		
	Color and Appearance	pH	Protein content by A280	RT (min)	Peak area (μV*sec)	Peak (μV)
A. Protein Stability prior to Freeze Thaw						
Day 0	2–8°C	Clear, colorless, particle free, homogeneous, non-viscous	7.42	0.231 mg/mL	ND	ND
	25°C	ND	ND	ND	ND	ND
	37°C	ND	ND	ND	ND	ND
Day 7	2–8°C	Clear, colorless, particle free, homogeneous, non-viscous	7.47	0.218 mg/mL	ND	ND
	25°C	Clear, colorless, particle free, homogeneous, non-viscous	7.47	0.212 mg/mL	ND	ND
	37°C	Clear, colorless, particle free, homogeneous, non-viscous	7.48	0.253 mg/mL	ND	ND
DAY 15	2–8°C	Clear, colorless, particle free, homogeneous, non-viscous	7.47	0.235 mg/mL	ND	ND
	25°C	Clear, colorless, particle free, homogeneous, non-viscous	7.47	0.230 mg/mL	ND	ND
	37°C	Clear, colorless, particle free, homogeneous, non-viscous	7.48	0.234 mg/mL	ND	ND
DAY 30	2–8°C	Clear, colorless, particle free, homogeneous, non-viscous	7.42	0.263 mg/mL	ND	ND
	25°C	Clear, colorless, particle free, homogeneous, non-viscous	7.41	0.272 mg/mL	ND	ND
	37°C	Clear, colorless, particle free, homogeneous, non-viscous	7.41	0.300 mg/mL	ND	ND
B. Protein Stability after Freeze-Thaw						
Freeze/Thaw 1	Clear, colorless, particle free, homogeneous, non-viscous	7.43	0.222 mg/mL	13.009	822293	16851
Freeze/Thaw 2	Clear, colorless, particle free, homogeneous, non-viscous	7.40	0.239 mg/mL	13.018	792084	15169
Freeze/Thaw 3	Clear, colorless, particle free, homogeneous, non-viscous	7.47	0.248 mg/mL	13.035	742475	13425

tested. Among these, Empigen® BB was determined to be the most effective at disrupting the *Na*-APR-1 (M74) aggregation observed in the QFF eluate while not interfering with the downstream chromatography or protein analysis methods. During process scale up, incorporation of Empigen® BB resulted in a significant increase in

the ratio of monomer relative to aggregate throughout the entire purification process and also improved the stability of the monomer at –80°C. Furthermore, the introduction of Empigen® BB throughout the purification process provided an additional benefit as it allowed for the reduction in the imidazole concentration

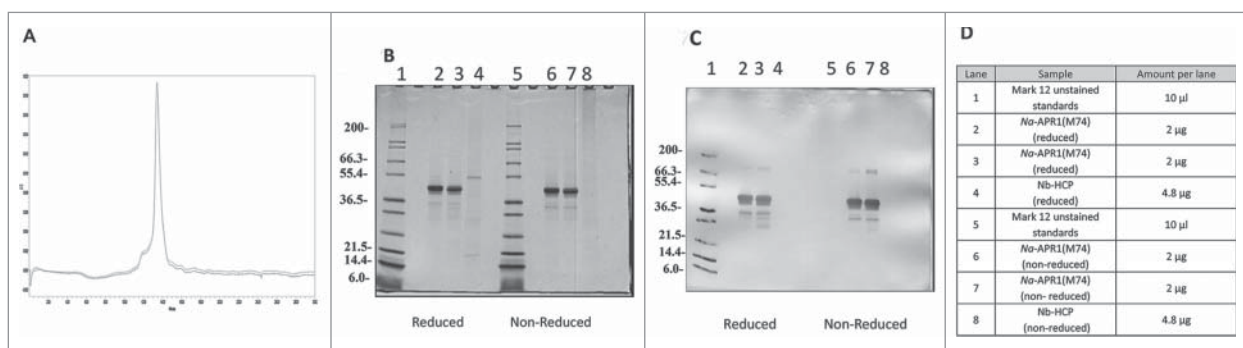


Figure 7. Evaluation of the purity profile of the *Na*-APR-1 (M74). (A): Analytical SEC HPLC chromatogram of the final purified *Na*-APR-1 (M74); (B): SDS-PAGE followed by Coomassie Blue Staining of *Na*-APR-1 (M74) and respective gel loading scheme (D), (C): Western Blot of *Na*-APR-1 (M74) probed with primary rabbit anti-WT-APR-1 antibodies and AP Goat Anti-Rabbit secondary antibodies and respective loading scheme (D); (D): Gel loading scheme for SDS-PAGE and Western Blot shown in (B and C).

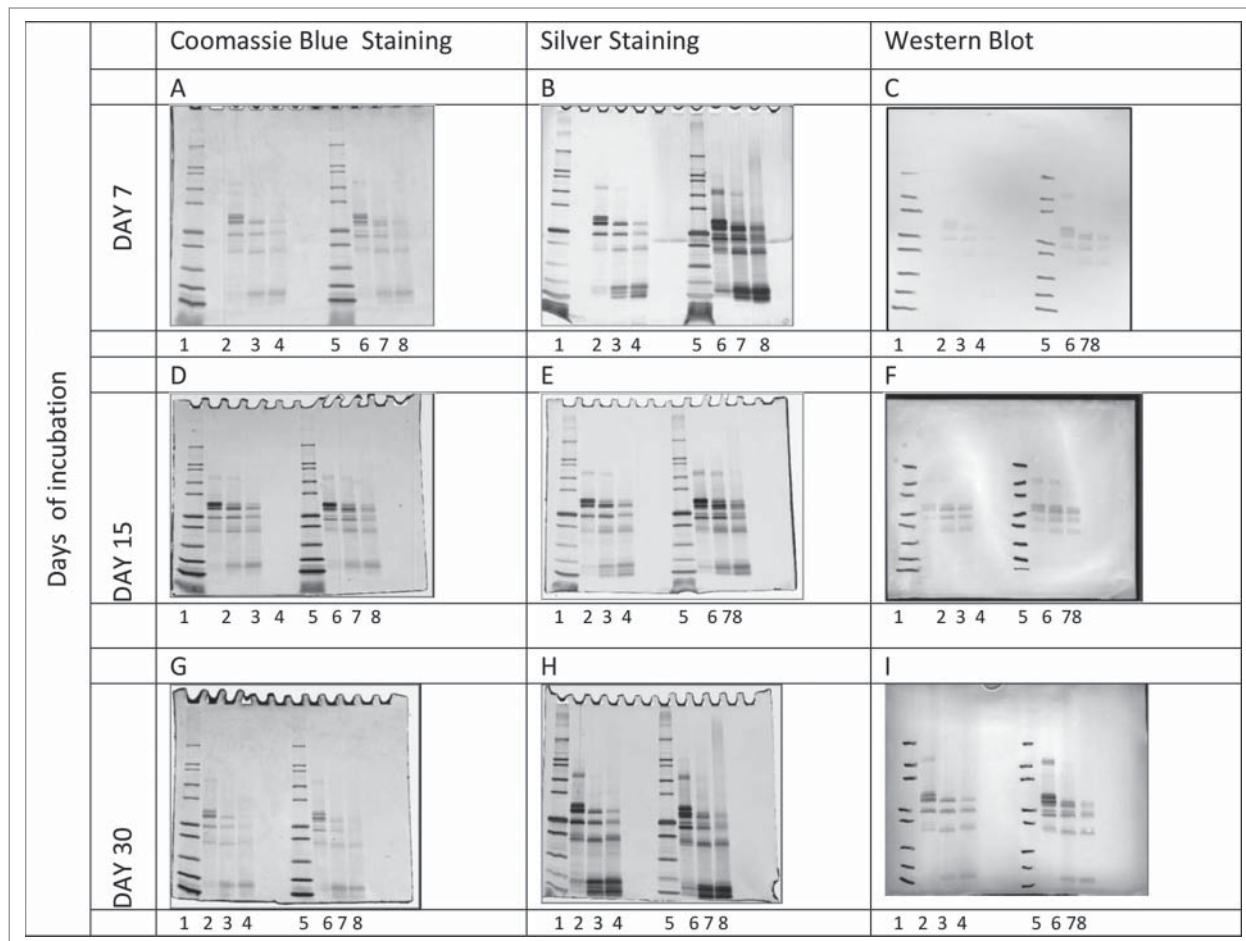


Figure 8. Electrophoretic analysis of *Na*-APR-1 (M74) samples collected during accelerated stability study. *Na*-APR-1 (M74) was analyzed by SDS-PAGE followed by Coomassie Blue staining (left column), silver staining (center column) and Western Blot (right column) probed with anti-APR-1 antibodies. *Na*-APR-1 (M74) was incubated at 2–8°C, 25°C or 37°C for 7 (A, B, C), 15 (D, E, F) and 30 days (G, H, I). Gels were loaded as follows. Lane 1: Mark 12™ unstained standards, lane 2: *Na*-APR1 (M74) (reduced) 2–8°C, lane 3: *Na*-APR1 (M74) (reduced) 25°C; lane 4: *Na*-APR1 (M74) (reduced) 37°C, lane 5: Mark 12™ unstained standards, lane 6: *Na*-APR1 (M74) (non-reduced) 2–8°C, lane 7: *Na*-APR1 (M74) (non-reduced) 25°C; lane 8: *Na*-APR1 (M74) (non-reduced) 37°C.

required for *Na*-APR-1 (M74) elution from the IMAC column from approximately 500 mM to 150 mM. This process change, as well as earlier modifications to the buffer salts, contributed to the evolution of a simple dilution of the IMAC eluate rather than performing a buffer exchange step prior to AEX chromatography which further streamlined the downstream process.

Despite these improvements, the overall yield of *Na*-APR-1 (M74) obtained after the final size exclusion chromatography step was only ~50% of the AEX recovery. This was attributed to the low resolution of the SEC chromatography and thus the difficulty in separating the pre- and main monomer fractions (M1 and M2, Fig. 4A). The main efforts of the work described was to isolate the *Na*-APR-1 (M74) monomer from the aggregate, as the monomer species has been the main focus of preclinical efficacy and safety studies. Monomer isolation was performed at a great sacrifice to the overall process recovery, but was deemed necessary since preliminary immunogenicity data from studies carried out in Balb/c mice indicated that the monomeric form once formulated with Alhydrogel® resulted in higher *Na*-APR-1

antibody titers than the aggregated form (data not shown). If the aggregate had been shown to be immunogenic, the improvement in overall SEC yield by combining the monomer with the aggregate would not be significant since the yield could not be greater than that of the preceding QFF step, or 17 ± 4 mg/kg biomass. Finally, from a manufacturing perspective, the presence of aggregates in the final product may be very problematic and difficult to control for batch to batch consistency. Therefore, efforts are currently ongoing to identify an alternative method to replace the size exclusion step in order to 1) reduce the overall manufacturing time, 2) reduce target protein loss while maintaining the same high purity, 3) ensure a better separation of monomeric and aggregated *Na*-APR-1 (M74) species without the loss observed using SEC, and 4) significantly improve the scalability of the *Na*-APR-1 (M74) manufacturing process. A simple terminal filtration process using a molecular weight cut off which would retain the aggregated species but allow the monomeric species to pass through is one option which can be explored. Such a change would significantly reduce manufacturing time,

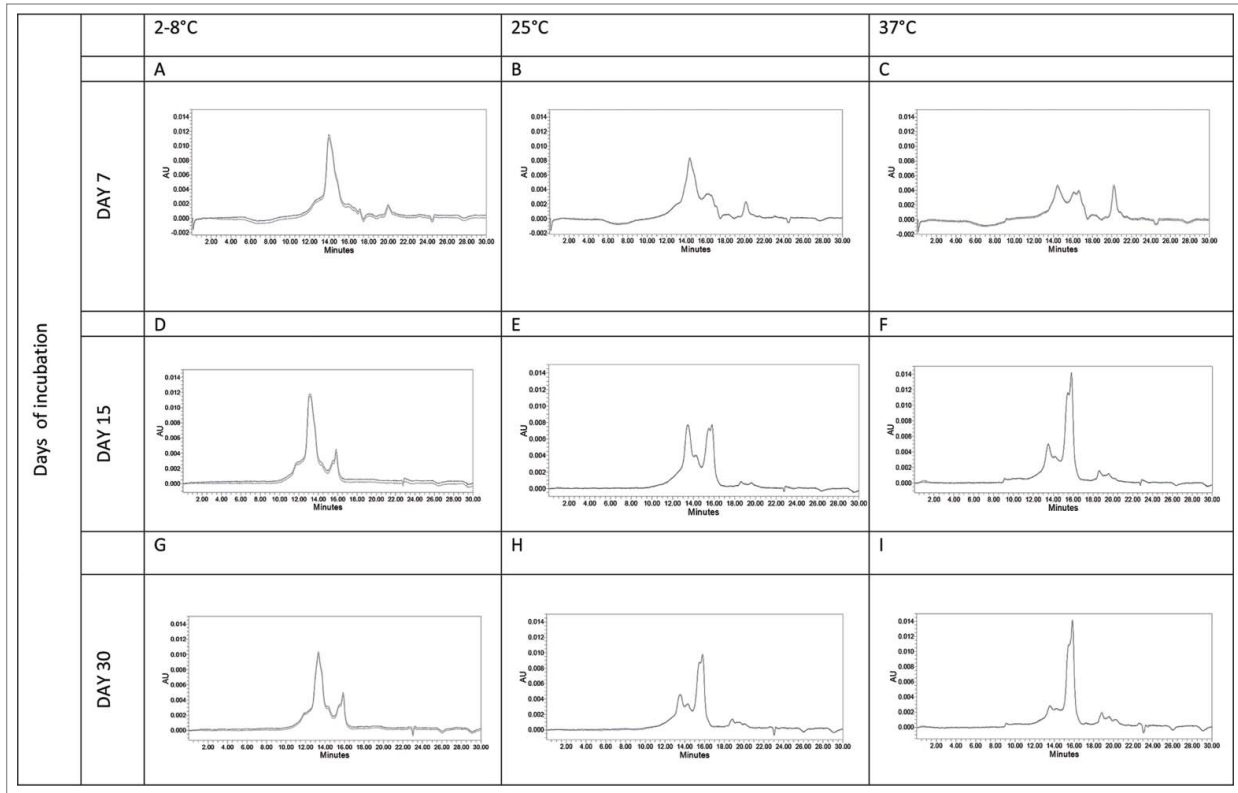


Figure 9. Analytical HPLC SEC of *Na*-APR-1 (M74) samples collected during accelerated stability studies. Samples were, incubated at 2–8°C, 25°C and 37°C for 7 (A, D, G), 15 (B, E,H) and 30 days (C, F, I).

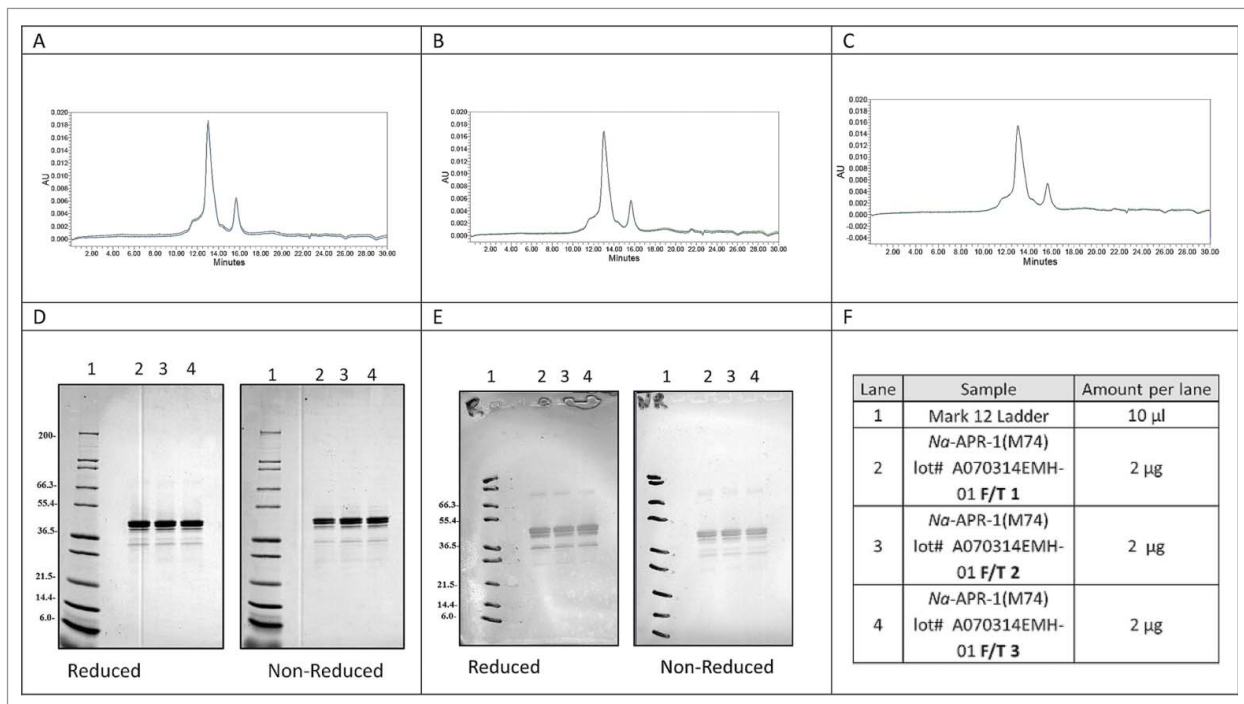


Figure 10. Freeze Thaw studies of *Na*-APR-1 (M74). A-C: Analytical HPLC SEC of *Na*-APR-1 (M74) after 3 (A, B, C) F/T cycles and reduced and non-reduced SDS-PAGE analysis followed by Coomassie Blue staining of the samples after 3 F/T cycles (D, Lane 2, and 4, respectively). Reduced and non-reduced western-blot analysis of the samples after 3 F/T cycles (E, lane 2 and 4, respectively), following the SDS-PAGE and western blot loading scheme defined in (E).

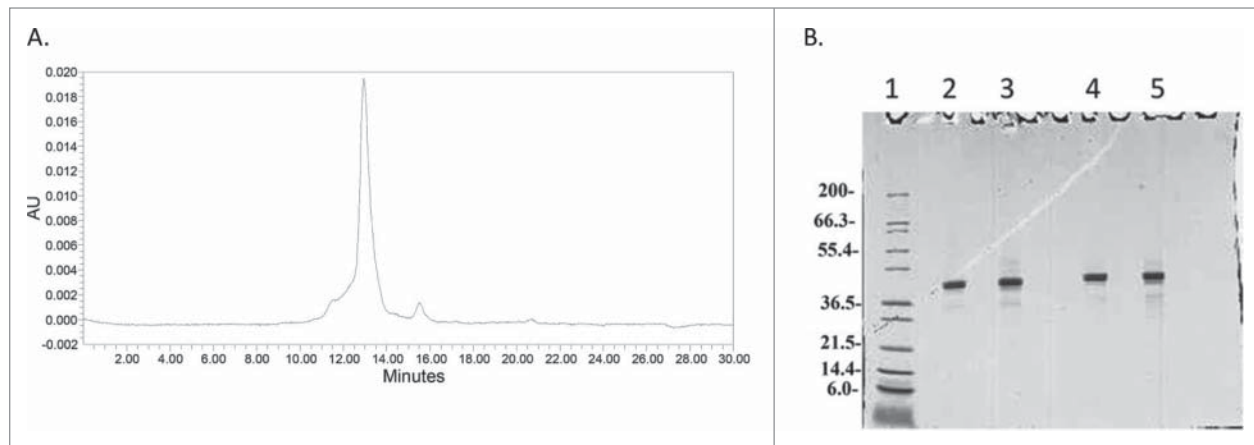


Figure 11. (A) Analytical HPLC SEC of *Na*-APR-1 (M74) after 24 month hold at -80°C and SDS-PAGE analysis followed by Coomassie Blue staining of *Na*-APR-1 (M74) after 24 months at -80°C . Lane 1 Mark12™ Marker, lane 2 and 3 *Na*-APR-1 (M74) non reduced, lanes 4 and 5 *Na*-APR-1 (M74) reduced.

cost, and eliminate the need to pool which is required for multiple SEC steps.

Based on the production process described herein, a final yield of *Na*-APR-1 (M74) of 8.6 ± 2.0 mg per kg plant biomass has been achieved. The process described has provided an initial platform for successfully transferring a *Na*-APR-1 (M74) production process to pilot scale manufacturing in support of ongoing clinical development. The final yield of *Na*-APR-1 (M74) per kg biomass after purification is, however; significantly lower when compared to other referenced vaccine candidate antigens expressed in *Nicotiana* (60–380 mg/kg biomass). This lower yield is likely due to 1) difficulty in expressing non-aggregated *Na*-APR-1 (M74) in most

systems which is likely due to the insoluble nature of the protein, and 2) the inclusion of a size exclusion chromatography step which reduces the final *Na*-APR-1 (M74) yield by 50% when compared to the yield at the previous QFF step. Due to the low yield of the current process, we have ongoing studies to investigate other strategies to improve yield by 1) exploring potential optimization steps to the current process, and 2) to re-evaluate the use of the *E. coli* expression system.

For the current plant-expressed product, several efforts are underway to elucidate the process weaknesses and implement improvements. Experiments have suggested that filters used in the current clarification procedures may retain target prior to the IMAC step. Use of membranes with alternate or low charge in combination with other extraction techniques may increase *Na*-APR-1 (M74) available for IMAC capture. In addition, Western blot analysis of the IMAC strip fraction has shown significant amount of protein remaining bound to the column after elution. Formation of *Na*-APR-1 (M74) aggregates trapped within the matrix of the resin or suboptimal elution conditions may both account for this loss. Furthermore, preliminary studies have suggested that the actual *Na*-APR-1 (M74) binding capacity of the QFF is greater than originally determined, thus allowing for a reduction in the QFF bed volume used, and consequently a reduction of the QFF eluate. This optimization should improve the overall QFF step recovery since protein concentration is being used as the pooling criteria. As previously suggesting, the use of Ultrafiltration (UF) after the QFF step rather than SEC could also broaden the acceptable fractions for pooling which would improve the overall product yield. However, because the final *Na*-APR-1 (M74) protein within the vaccine is to be without a tag, significant improvement to the overall expression level will be required. If we are successful in improving the yield using the plant system to the point where we can use a tagless version of *Na*-APR-1 (M74), then pre-clinical bridging studies will need to be performed to confirm potency and equivalency to the original HIS-tagged version. There are however additional hurdles which must be resolved by industry in general for production of pharmaceutical proteins in plant systems.³⁴ For example, unlike bacterial and mammalian expression systems, the nature and safety of potentially

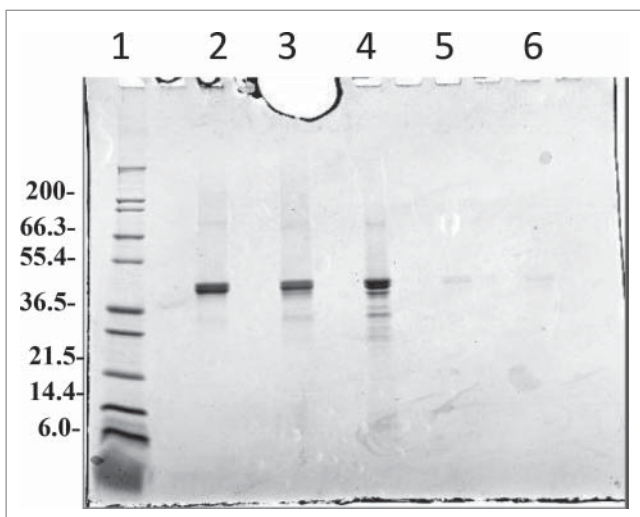


Figure 12. SDS-PAGE analysis followed by Coomassie Blue staining of the *Na*-APR-1 (M74) pellet and supernatant fractions after desorption from the Alhydrogel® adjuvant. After storage for 37 months at $2-8^{\circ}\text{C}$, the vaccine was treated with desorption buffer and separated into a pellet (lanes 2 and 3) and supernatant (lanes 5 and 6) fraction by centrifugation. Lane 1, Mark12™ molecular weight standards, lane 2 and lane 3 non-reduced pellet of *Na*-APR-1 (M74) after desorption, lane 4 non-reduced *Na*-APR-1 (M74) protein controls, lanes 5 and 6 non-reduced supernatant of *Na*-APR-1 (M74) once desorbed from the Alhydrogel®.

contaminating proteins has not been well established. In addition, Industry standards concerning plant strains and methods of cultivation must be identified. Also, robust downstream processes must be identified and standardized since processing of plant material is unique when compared to other expression systems. However, these challenges have not slowed the drive to produce vaccines and biotherapeutics under GMP in plants as reviewed in the same article.³⁴ Specifically for transiently expressed plant produced recombinant proteins, a number of facilities have opened in the last 2 decades to drive these processes at manufacturing scale. To address upstream challenges in plant production, Fraunhofer Center for Molecular Biotechnology (Newark, DE) has built a fully automated cGMP facility for pilot-scale production of transiently expressed protein. This facility handles all aspects from seeding to harvest in a nearly lights-off manner.³⁵ Concerns of containment and compatibility of using plants for cGMP process has been advanced by tightly controlled and contained indoor environments.³² In addition to the Fraunhofer facility, the large-scale plant expressed protein manufacturers of Kentucky BioProcessing (Owensboro, KY), Medicago Inc. (North Carolina) and Caliper Biotherapeutics (College Station, Texas) all have manufacturing facilities with various levels of robotic and flexible use facilities. To address the hurdle of generating industry standards, US regulatory bodies (FDA, USDA and APHIS) have drafted guidelines describing unique aspects of plant produced pharmaceuticals, the responsibilities of regulatory agencies and their expectations of manufacturer's in this area.³⁶ The draft guidance includes the use of plant transient expression systems, reviews expectations for characterizing and maintaining host plants and generally guides the manufacturer to the same Federal Regulations applied to any biological intended for human use.

A second option is to re-explore the use of the *E. coli* expression system and test several new strategies to achieve a soluble *Na*-APR-1 (M74) product which include 1) use of high throughput screening tools to screen a wider array of different buffer conditions to identify optimal salts and buffer conditions to achieve greater solubility and stability, 2) testing alternative *E. coli* vectors and host strains, and 3) consider addition of the native pro sequence since recent data suggests that this sequence may be necessary to achieve a soluble product. However as with the above scenario in the plant system, switching expression systems would also require bridging studies be performed to show potency and equivalency to the plant derived *Na*-APR-1 (M74) which was used for the Phase 1 studies.

Characterization of the *Na*-APR-1 (M74) protein and accelerated stability studies were performed during the product development cycle for a number of reasons, including the establishment of release criteria and storage conditions, and the development of a formulation which best stabilized the product. In addition, accelerated studies represent a means to ensure that the analytical methods selected for release are stability indicating assays. Freezing of protein biologics is often included in the manufacturing processes as it allows prolonged storage over time. The impact of freezing, thawing and cold storage over a prolonged time however may be protein dependent; therefore, a stability study was designed to include freeze/thaw cycles. The goal of stability evaluation for a bulk intermediate is to establish whether the material

can be maintained at a specified temporary storage condition (for example at 2–8°C) for a given period of time without a negative impact on the quality of the final product. In addition, it is important to demonstrate that storage of the purified protein has negligible impact on the final product stability. The ongoing stability studies have shown that the purified *Na*-APR-1 (M74) has maintained its biochemical features when stored at –80°C, with no signs of degradation or aggregation up to 24 months. Accelerated stability conditions were used to increase the rates of degradation and enhance the likelihood of observing significant differences in the stressed sample compared to the unstressed sample. Development of analytical methods able to detect these differences was essential to monitor both degradation and aggregation events during the long term stability testing of the protein.

The stability data collected to date indicate that the *Na*-APR-1 (M74) is stable at 2–8°C only for approximately 7 days after which signs of degradation were observed. Although, the pH and the overall A_{280} were not significantly affected by the 7 days hold, SDS-PAGE analysis indicated a reduction in density of the 42 kDa band corresponding to *Na*-APR-1 (M74) as well as the appearance of low molecular weight degradation products. These data indicate that any refrigerated hold times during post-manufacture must be kept to a minimum. The degradation observed was more rapid at the higher temperature tested, but was also evident when the material was stored at 2–8°C for period of times longer than 7 days. In just 7 days, at 25°C or 37°C the density of the main bands corresponding to the *Na*-APR-1 (M74) protein decreased from 80 to 10%. Increase in aggregation was not observed during the length of this study. Similar protein degradation was observed by SEC-HPLC, supporting the use of both SDS-PAGE and SEC-HPLC assay methodologies for monitoring stability. Taken together, these data also support the conclusion that the final *Na*-APR-1 (M74) protein can only be held at elevated temperatures for a short time without risking a detrimental impact on the integrity of the protein. In contrast, the stability of the protein was maintained when the samples were stored at –80°C for longer periods of times. As indicated by the ongoing long-term stability studies, the protein has maintained its properties for all time points up to 2 years.

In order for the vaccine to be effective, the use of immunostimulatory agents (or adjuvants) is critical for most recombinant protein vaccines because proteins are usually weakly immunogenic. The most widely used vaccine adjuvants are aluminum salts and there is extensive data regarding the safety of these adjuvants. The most common are aluminum phosphate (Adju-Phos[®]) and aluminum hydroxide (Alhydrogel[®]). The adsorption of a protein on aluminum compounds heavily depends on electrostatic forces between the protein and the adjuvant. Based on the pI of *Na*-APR-1 (M74) as well as the pH and salts present in the formulation buffer, Alhydrogel[®] was selected as the most suitable adjuvant in order to maximize the electrostatic interaction between the aluminum salt and the protein. Binding studies and a Langmuir binding isotherm further supported the rationale for the Aluminum/protein ratio of the vaccine formulation. Ongoing stability studies have validated this selection since the formulated product, i.e., the protein bound to Alhydrogel[®], has

been shown to maintain its properties at the intended storage conditions of 2–8°C so far for up to 3 years. No variation in pH, in protein content and percentage of protein adsorbed to Alhydrogel[®], or protein identity has been detected during this time. The long term stability of both the *Na*-APR-1 (M74) protein as well as the formulated product is to be monitored for a minimum of 5 years. The implementation of optimal storage and formulation protocols based on the described stability data has allowed the Sabin PDP to successfully move *Na*-APR-1 (M74) formulated with Alhydrogel[®] into phase 1 clinical testing.

Materials and Methods

Cloning of *Na*-APR-1 (M74) into launch vector and expression in *N. benthamiana*

The DNA sequence encompassing amino acids 74–446 of the *N. americanus* Aspartic Protease-1 (*Na*-APR-1, NCBI accession number CAC00543.1), without the pro-sequence, was optimized for expression in plants and synthesized by Genart. The synthesized sequence was cloned into the Tobacco Mosaic virus (TMV)-based launch expression vector pGRD4^{23,24} with a PR-1a signal peptide of *Nicotiana tabacum* (BAA14220) replacing the native *Na*-APR-1 signal peptide at the N-terminus.²⁵ A His tag (6xHIS), as well as ER retention sequence (KDEL), was inserted at the C-terminus (Fig. 1) of the protein to aid purification. Additional modifications to the *Na*-APR-1 (M74) sequence included the D113A and D300A mutations to eliminate the hemoglobinase activity. The resulting construct and the helper plasmid, pSoup, which provides replication functions in trans, were then introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation in order to generate the Research Cell Bank.

Preparation of Master and Working Cell Banks for *Agrobacterium tumefaciens* (GV3101) *Na*-APR-1 (M74)

Master and Working Cell Banks (MCB/WCB) of *Agrobacterium tumefaciens Na*-APR1 (M74) were prepared following cGMP practices as follows: For production of the Master Cell Bank, a vial of Research Cell Bank was thawed and used to inoculate 1L of alternative protein source LB broth containing Kanamycin (50 µg/ml). The culture was grown at 28 + 1°C for 16–24 hours. The culture was centrifuged and re-suspended in media to an OD₆₀₀ of >5 and glycerol was added to a final concentration of 20–25% to create the Master Cell Bank. Aliquots of the Master Cell Bank glycerol stocks were prepared in cryovials at 1.0 mL per vial and stored at ≤–70°C. A vial of the MCB was thawed and used to create the WCB using the same procedure as described above.

Preparation of *Agrobacterium* and infiltration

One vial of WCB was thawed and used to inoculate a 2L shake flask containing 250 mL sterile LB medium with 50 µg/mL Kanamycin. Fermentation was performed at 28°C. Air was supplied and agitation was gradually increased to maintain the O₂ saturation. The pH was maintained at 7.0. Directly after inoculation of the fermenter, a glucose feed was initiated to maintain the

carbohydrate source. Fermentation was carried out for at least 15 hours. Cell density was monitored during fermentation and the run was terminated when the OD₆₀₀ reached a cell density greater than 5. For dilution of the *Agrobacterium* and infiltration, Milli-Q water was added to the culture to reduce the cell density to OD₆₀₀ of 0.5 (range 0.3–1.0). Hydroponically grown 29–32 day old *N. benthamiana* seedlings were vacuum infiltrated with the *Agrobacterium* as described previously.^{24,26} Briefly, the plant trays were drained and manually inverted into the diluted *Agrobacterium* solution. After vacuum infiltration the plants were submerged in a tank with Milli-Q water for rinsing and subsequently grown for 6 days to accumulate recombinant *Na*-APR-1 (M74). On day 5, approximately 50 g of plant biomass was sampled to determine the IMAC recovery of target protein per gram of plant tissue (based on SDS-PAGE, data not shown)

Plant harvesting, dicing, homogenization and clarification prior to chromatography

N. benthamiana plants transiently expressing *Na*-APR-1 (M74) were harvested at 6 days post-infiltration by clipping at the base of the stem. Aerial plant tissue of the post-infiltration plants were then collected and separated from the trays. Approximately 5 kg of tissue was used for *Na*-APR-1 (M74) extraction at a 5 kg scale. The aerial tissue was diced, mixed with extraction buffer and passed twice through a Comitrol 1500 homogenizer. Triton X-100 was added to 0.5% and the homogenate mixed for 20 minutes at 4°C. The majority of solids were removed from the Triton extracted homogenate by continuous flow disk stack centrifugation (12,000 rpm). Residual solids were removed by passage first through a 0.27 m² depth filter (3M Cuno Zeta Plus series) followed by a 0.2 µm dead end filter (3M Cuno LifeAssure PDA series). Immobilized Metal Affinity Chromatography (IMAC) load buffer was used as a filter chase (~10% total homogenate volume) and the resulting clarified extract was held in a sterile bioprocess bag (Sartorius Stedim, Flexboy) at 2–8°C until chromatography.

Purification of *Na*-APR-1 (M74)

Na-APR-1 (M74) purification consisted of 3 column chromatography steps: first an Immobilized Metal Affinity Chromatography (IMAC) purification step was performed in order to capture the *Na*-APR-1 (M74) from the crude plant extraction supernatant. The IMAC eluate was then purified using a Q Sepharose FF anion exchange column (AEX), followed by a Superdex 200 (size-exclusion) column. IMAC purification was performed at 2–8°C. An IMAC column (7 × 22 cm packed bed) with a bed volume of 843 mL resin was first washed with Milli-Q water followed by equilibration with 3 column volumes (CV) of 50 mM Tris, 0.5 M Sodium Chloride, 20 mM Imidazole, 0.5% Triton X-100, pH 7.0 (Capture Buffer A1) at a flow rate of 240 cm/hr. After equilibration with Capture Buffer A1, the clarified harvest was loaded at 240 cm/hr. onto the IMAC column followed by washing with 4 CV of Capture Buffer A1 at 240 cm/hr. to allow residual, unbound material to flow through. The IMAC column was then washed with a minimum of 5 CV of 10 mM Tris, 0.1 M Sodium Borate, 0.3% Empigen BB, pH

8.0 (Capture Buffer A2) at a flow rate of 335 cm/hr. until the absorbance at 280 nm (A_{280}) returned to a stable baseline. Finally, the *Na*-APR-1 (M74) was eluted from the column with approximately 2 CV of 10 mM Tris, 0.1 M Sodium Borate, 150 mM Imidazole, 0.3% Empigen BB, pH 8.0 at a flow rate of 218 cm/hr. while monitoring the A_{280} . The IMAC elution fractions containing *Na*-APR-1 (M74) was stored at 2–8°C. A Q Sepharose FF (GE Healthcare) column ($9 \pm 3 \times 7$ cm) with a packed to bed volumes of 238–423 mL of resin was sanitized with a 2.5 CV wash of 0.5 N Sodium Hydroxide (NaOH) followed by a 60 minute hold and an additional 2.5 CV wash of 0.5 N NaOH. Residual base was removed from the column with a 9 CV Milli-Q water wash. After sanitization, the column was stored in 20% ethanol until use. Prior to chromatography, the resin was washed with Milli-Q water, equilibrated with 3 CV of 10 mM Tris, 60 mM Sodium Borate, 560 mM Sodium Chloride, 0.3% Empigen BB, pH 8.0 (Q Solution C) and then equilibrated with 5 CV of 10 mM Tris, 100 mM Sodium Borate, 0.3% Empigen BB, pH 8.0 (Q Solution A). Prior to loading the column, the *Na*-APR-1 (M74) containing IMAC eluent was equilibrated to room temperature and an equal volume of Q Solution A was added to ensure the conductivity was <17 mS/cm. The sample conductivity and pH were then confirmed to be within acceptable ranges of 16.5–19.0 mS/cm and 7.8–8.4, respectively. The diluted IMAC eluate was loaded onto the Q Sepharose FF column for a targeted 4 minute media contact time. After loading, the resin was washed with at least 5 CV of Q Solution A followed by 5 CV of 10 mM Tris, 96 mM Sodium Borate, 56 mM Sodium Chloride, 0.3% Empigen BB, pH 8.0 (Q Solution B). Finally the *Na*-APR-1 (M74) was eluted using Q Solution C while monitoring the A_{280} . Individual elution fractions were analyzed by SDS-PAGE. Fractions which were enriched with *Na*-APR-1 (M74) (> 1.8 mg/mL) were pooled and stored overnight at 2–8°C prior to SEC.

SEC (Superdex 200)

SEC provides a polishing step (removal of high molecular weight species) as well as a buffer exchange step for the QFF ion exchange elution pool. SEC was performed at room temperature over tandem Superdex S200 Prep grade columns (50×30.5 cm, GE Healthcare) packed to a total bed volume of 1215 mL. The columns were sanitized with 1.0 N NaOH at a flow rate of 31 cm/hr., washed with Milli-Q water at the same flow rate, then equilibrated with 10 mM Imidazole, 150 mM Sodium Chloride, 0.3% Empigen BB, pH 7.4 (SEC running buffer) for 4 CV overnight. The *Na*-APR-1 (M74) pool eluate from the Q Sepharose FF column was divided into 2 or 3 equal aliquots based on the total volume. Each aliquot was processed separately on the Superdex S 200 column using the same process parameters as described above, and separating each run with an equilibration step. For each run, one aliquot was loaded onto the pre-equilibrated Superdex S200 column (3.4–4.0% bed volume) and allowed to flow through the column in SEC running buffer while monitoring the A_{280} of the eluate. The eluate was collected in fractions comprising 5 main elution sections: an aggregate upslope (A1), an aggregate downslope (A2), a pre-monomer

peak (M1), the main monomer peak (M2), and monomer tail (MT). Fractions enriched for *Na*-APR-1 (M74) were stored overnight at 2–8°C until pooling. Pooling provided a means of excluding high molecular weight contaminants from the leading side of the main monomer peak (M2) while maintaining a target concentration of ≥ 0.2 mg/mL, as determined by UV_{280} , for the trailing side of the M2 peak. The final pooled product was sterile filtered through a 0.22 μ m filter, aliquoted into sterile Nalgene cryovials, and frozen at $\leq -70^\circ\text{C}$. The final *Na*-APR1 (M74) concentration was determined by densitometry of *Na*-APR1 (M74) bands after SDS-PAGE and Coomassie Blue staining and comparison to BSA standards, and by absorption at 280 nm ($A_{0.1\%} = 1.16$) for the final purified protein.

SDS-PAGE/Western Analysis

For the *Na*-APR-1 (M74) samples collected during purification, SDS-PAGE analysis was performed on 10% acrylamide gels stained with Coomassie (Gel Code Blue, Pierce Rockford, IL). Western blot analysis on the same samples was performed as follow: SDS-PAGE separated samples were transferred to PVDF membranes, blocked with I-Block (Applied Biosystems, Carlsbad, CA) and developed using either anti-4X HIS (Qiagen, Valencia, CA) or rabbit anti-*Na*-APR-1 (M74) antibodies. Excess primary antibodies were removed by washing the blots with 1xPBS containing 0.1% Tween 20 (PBST) followed by an anti-rabbit or mouse HRP conjugated secondary antibody (1:10,000 dilution, Jackson ImmunoResearch, West Grove, PA) and by detection with a chemiluminescent substrate (SuperSignal West Pico, Thermo Fisher). Images were captured using either a flat-bed scanner or a Genome chemiluminescence detector (Syngene Corp.). For characterization of the purified protein, the presence of residual host cell proteins was assessed by SDS-PAGE and Western blot analysis on reduced and non-reduced 4–20% Tris-glycine gels. A mouse primary antibody recognizing host cell proteins was used to detect the presence of host contaminants at a 1:3000 dilution. Alkaline phosphatase conjugated goat anti rabbit IgG and goat anti mouse secondary antibodies (KPL) were used for colorimetric detection at 1:3000 or 1:10000 dilution, respectively. The blots were developed using a BCIP/NBT (KPL) membrane phosphatase substrate system. 4–20% Tris glycine gels and 4–12% Bis-Tris gels were used for evaluation of sample purity and electrophoresis was performed following the manufacturer's recommendations. For the reduced samples, β -mercaptoethanol was added to the 2X Tris-glycine sample-loading buffer to a final concentration of 10% and to the 4X Bis-Tris sample-loading buffer to a final concentration of 20%. Approximately 2 μ g of non-reduced or reduced protein was loaded for each lane. Gels were stained, de-stained and scanned as described above. Identity of the protein was confirmed by Western blot with target specific antibodies raised in rabbits and used to detect *Na*-APR-1 (M74) at a 1:1000 dilution.

Analysis by SEC

SEC analysis of in process samples was performed using a Superdex 200 10/300 GL column with effluent monitored at A_{280} . Samples were injected with a 50% sample loop overfill at a

flow rate of 0.5 mL/min. The running buffer used was PBS (pH 7.4) supplemented with 0.3% Empigen BB.

Purity by SEC-HPLC

Purity of stability samples was analyzed by SEC-HPLC. All samples were injected in a 50 μ L volume at a flow rate of 0.6 mL/min onto a Tosoh TSK gel G3000SWxl column using a Shimadzu Prominence UFLC with a PDA detector. The column was run at room temperature at a flow rate of 0.6 mL/min for 30 min, using 1X PBS + 0.3% Empigen[®] BB pH 7.4 as the mobile phase. To determine the percent purity, the area of the main peak was compared to the area of all other peaks on the chromatograph (excluding sample buffer peaks).

Freeze/thaw studies of the *Na*-APR-1 (M74) protein

Three 1 mL aliquots of *Na*-APR-1 (M74) were placed in liquid nitrogen for 10 minutes followed by a thaw step at room temperature. The three vials were exposed respectively to 1, 2 or 3 freeze/thaw cycles. After each cycle, samples were tested for color and appearance by visual inspection on both a white and black background, for pH by a Thermo Scientific Orion Versa Star[™] pH meter using standard manufacturer recommendations, and for protein content by the absorbance at 280 nm (Ultrospec 6300pro). The identity, and purity of the sample was also tested by SEC-HPLC using a Waters Alliance[®] HPLC system with the Empower[®] 2 software for data analysis. 50 μ g of *Na*-APR-1 (M74) at 1 μ g/ μ L were loaded on a TSK gel G3000SWxl column with 1X PBS + 0.3% Empigen[®] BB pH 7.4 as the mobile phase and at a flow rate of 0.6 mL/min and run time of 30 min. SDS-PAGE analysis was performed using 4–20% Tris-glycine gels with a protein load of 2 μ g of each sample per lane under both reducing and non-reducing conditions. Gels were stained with Coomassie Blue or Silver Stain following conventional procedures.

Accelerated and long-term stability studies of the *Na*-APR-1 (M74) protein

For the accelerated stability studies, the *Na*-APR-1 (M74) protein was placed at 3 different temperatures, 2–8°C, 25°C and 37°C for a maximum time period of 30 days and then characterized. At time zero, 1 mL protein aliquots were placed at the above specified temperatures and analyzed after 7, 15, and 30 days for color and appearance, pH measurement, and protein content by absorbance at 280 nm, as performed for freeze/thaw analysis. Identity and purity by SEC-HPLC, as well as identity by SDS-PAGE followed by Coomassie Blue staining and silver staining, were also performed following the same procedures as described above. For long term stability studies, the *Na*-APR-1 (M74) protein was maintained at its storage temperatures of –80°C and then characterized after 3, 6, 9, 12, 18, 24 months and every year thereafter, for a total of 5 years (ongoing). Color and appearance, pH measurement, and protein content by absorbance at 280 nm, Identity and purity by size exclusion HPLC, as well as identity by SDS-PAGE followed by Coomassie Blue staining was performed at each time point following the same procedures as described above.

Preparation of the *Na*-APR-1 (M74) formulation

The *Na*-APR-1 (M74) protein stock solution and the Alhydrogel[®] were combined in 10 mM imidazole, 150 mM Sodium Chloride buffer, 0.3 % Empigen[®] BB, pH of 7.4 such that the final protein concentration was 0.1 mg/mL and the final Alhydrogel[®] concentration was 0.8 mg/mL. Upon formulation, adsorption of the protein to the Alhydrogel[®] surface was assessed by SDS-PAGE and OPA assay.²⁷

Stability testing of the formulated protein

Stability of the *Na*-APR-1 (M74)/Alhydrogel[®] formulation was monitored 1, 3, 6, 9, 18, 24, 37 months post formulation. Color and appearance, pH measurement, protein content by OPA and identity by SDS-PAGE after desorption with 100 mM sodium citrate, 92 mM dibasic sodium phosphate, pH 8.9 followed by Coomassie Blue staining were evaluated at each time point. While the color and appearance and pH were evaluated as previously described, the OPA assay was used to quantify the total protein content of the vaccine. The OPA assay was performed using a Victor 3 multilabel counter with Wallac 1420 software (PerkinElmer). A series of dilutions of non-formulated *Na*-APR-1 (M74) protein ranging from 0 to 250 μ g/mL were made in PBS pH 7.4 as the diluent and used to populate a standard curve. Over this range, the fluorescence intensity increased in a linear fashion with the concentration of the protein. 40 μ L of each test sample and standard was dispensed into a 96 wells microplate to which 200 μ L of OPA reagent solution was added. Samples were incubated for approximately 2 minutes with moderate shaking at room temperature and the fluorescence signal was measured using a Victor 3 multilabel counter plate reader with a 355 nm, 40 bandwidth, excitation filter and a 460 nm, 40 nm bandwidth, emission filter. The standard curve following the least means squared linear equation was generated using the Wallac 1420 software. The same assay was used to evaluate the percent protein adsorbed to Alhydrogel[®].

Disclosure of Potential Conflicts of Interest

All authors of this manuscript currently are involved in a program for the development of human hookworm vaccine. There are no other conflicts of interest to disclose.

Acknowledgments

The authors would like to thank Joey Norikane, Rebecca Snow, Moneim Shamloul, Ruben Lopez, Shireen Sheikh, and Amy Rhee from Fraunhofer USA Center for Molecular Biotechnology (FhCMB; Newark, DE) for their technical support with the work described in this manuscript.

Funding

This work is supported by the Sabin Vaccine Institute through grants obtained from the Bill & Melinda Gates Foundation (Grant #32472 and #38988) and the Dutch Ministry of Foreign Affairs.

References

- Hotez PJ, Alvarado M, Basanez MG, Bolliger I, Bourne R, Boussinesq M, Brooker SJ, Brown AS, Buckle G, Budke CM, et al. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. *PLoS Negl Trop Dis* 2014; 8:e2865; PMID:25058013; <http://dx.doi.org/10.1371/journal.pntd.0002865>
- Pullan RL, Smith JL, Jasrasaria R, Brooker SJ. Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. *Parasit Vectors* 2014; 7:37; PMID:24447578; <http://dx.doi.org/10.1186/1756-3305-7-37>
- Hotez PJ, Brooker S, Bethony JM, Bottazzi ME, Loukas A, Xiao S. Hookworm Infection. *N Engl J Med* 2004; 351:799–807; PMID:15317893; <http://dx.doi.org/10.1056/NEJMra032492>
- Kassebaum NJ, Jasrasaria R, Naghavi M, Wulf SK, Johns N, Lozano R, Regan M, Weatherall D, Chou DP, Eisele TP, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood* 2014; 123:615–24; PMID:24297872; <http://dx.doi.org/10.1182/blood-2013-06-508325>
- Smith JL, Brooker S. Impact of hookworm infection and deworming on anaemia in non-pregnant populations: a systematic review. *Trop Med Int Health* 2010; 15:776–95; <http://dx.doi.org/10.1111/j.1365-3156.2010.02542.x>
- Brooker S, Hotez PJ, Bundy DA. Hookworm-related anaemia among pregnant women: a systematic review. *PLoS Negl Trop Dis* 2008; 2:e291; PMID:18820740; <http://dx.doi.org/10.1371/journal.pntd.0000291>
- Ranjit N, Zhan B, Hamilton B, Stenzel D, Lowther J, Pearson M, Gorman J, Hotez P, Loukas A. Proteolytic degradation of hemoglobin in the intestine of the human hookworm *Necator americanus*. *J Infect Dis* 2009; 199:904–12; PMID:19434933; <http://dx.doi.org/10.1086/597048>
- Williamson AL, Brindley PJ, Knox DP, Hotez PJ, Loukas A. Digestive proteases of blood-feeding nematodes. *Trends Parasitol* 2003; 19:417–23; PMID:12957519; [http://dx.doi.org/10.1016/S1471-4922\(03\)00189-2](http://dx.doi.org/10.1016/S1471-4922(03)00189-2)
- Pearson MS, Bethony JM, Pickering DA, de Oliveira LM, Jariwala A, Santiago H, Miles AP, Zhan B, Jiang D, Ranjit N, et al. An enzymatically inactivated hemoglobinase from *Necator americanus* induces neutralizing antibodies against multiple hookworm species and protects dogs against heterologous hookworm infection. *FASEB J* 2009; 23:3007–19; PMID:19380510; <http://dx.doi.org/10.1096/fj.09-131433>
- Loukas A, Bethony JM, Mendez S, Fujiwara RT, Goud GN, Ranjit N, Zhan B, Jones K, Bottazzi ME, Hotez PJ. Vaccination with recombinant aspartic hemoglobinase reduces parasite load and blood loss after hookworm infection in dogs. *PLoS Med* 2005; 2:e295; PMID:16231975; <http://dx.doi.org/10.1371/journal.pmed.0020295>
- Xiao S, Zhan B, Xue J, Goud GN, Loukas A, Liu Y, Williamson A, Liu S, Deumic V, Hotez P. The evaluation of recombinant hookworm antigens as vaccines in hamsters (*Mesocricetus auratus*) challenged with human hookworm, *Necator americanus*. *Exp Parasitol* 2008; 118:32–40; PMID:17645877; <http://dx.doi.org/10.1016/j.exppara.2007.05.010>
- Zhan B, Liu S, Perally S, Xue J, Fujiwara R, Brophy P, Xiao S, Liu Y, Feng J, Williamson A, et al. Biochemical characterization and vaccine potential of a heme-binding glutathione transferase from the adult hookworm *Ancylostoma caninum*. *Infect Immun* 2005; 73:6903–11; PMID:16177370; <http://dx.doi.org/10.1128/IAI.73.10.6903-6911.2005>
- Zhan B, Perally S, Brophy PM, Xue J, Goud G, Liu S, Deumic V, de Oliveira LM, Bethony J, Bottazzi ME, et al. Molecular cloning, biochemical characterization, and partial protective immunity of the heme-binding glutathione S-transferases from the human hookworm *Necator americanus*. *Infect Immun* 2010; 78:1552–63; PMID:20145100; <http://dx.doi.org/10.1128/IAI.00848-09>
- Curti E, Seid CA, Hudspeth E, Center L, Rezende W, Pollet J, Kwityn C, Hammond M, Matsunami RK, Engler DA, et al. Optimization and revisions of the production process of the *Necator americanus* glutathione S-transferase 1 (Na-GST-1), the lead hookworm vaccine recombinant protein candidate. *Hum Vaccin Immunother* 2014; 10; PMID:25424799; <http://dx.doi.org/10.4161/hv.28872>
- Goud GN, Deumic V, Gupta R, Brelford J, Zhan B, Gillespie P, Plietskatt JL, Tsao EI, Hotez PJ, Bottazzi ME. Expression, purification, and molecular analysis of the *Necator americanus* glutathione S-transferase 1 (Na-GST-1): a production process developed for a lead candidate recombinant hookworm vaccine antigen. *Protein Exp Purif* 2012; 83:145–51; PMID:22503665; <http://dx.doi.org/10.1016/j.pep.2012.03.013>
- Leuzinger K, Dent M, Hurtado J, Stahnke J, Lai H, Zhou X, Chen Q. Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. *J Vis Exp* 2013; PMID:23913006
- Chichester JA, aY V. Plants as Alternative Systems for Production of Vaccines. *Human vaccines* 2007; 3:146–9; PMID:17643065
- Mett V, Farrance CE, Green BJ, Yusibov V. Plants as bio-factories. *Biologicals* 2008; 36:354–8; PMID:18938088; <http://dx.doi.org/10.1016/j.biologicals.2008.09.001>
- Rybicki EP. Plant-made vaccines for humans and animals. *Plant Biotechnol J* 2010; 8:620–37; PMID:20233333; <http://dx.doi.org/10.1111/j.1467-7652.2010.00507.x>
- Giddings G. Transgenic Plants as protein factories. *Curr Opin Biotechnol* 2001; 12:450–4; PMID:11604319; [http://dx.doi.org/10.1016/S0958-1669\(00\)00244-5](http://dx.doi.org/10.1016/S0958-1669(00)00244-5)
- Mett V, Lyons J, Musiyuchuk K, Chichester JA, Brasil T, Couch R, Sherwood R, Palmer GA, Streatfield SJ, Yusibov V. A plant-produced plague vaccine candidate confers protection to monkeys. *Vaccine* 2007; 25:3014–7; PMID:17287055; <http://dx.doi.org/10.1016/j.vaccine.2007.01.017>
- Shoji Y, Chichester JA, Bi H, Musiyuchuk K, de la Rosa P, Goldschmidt L, Horsey A, Ugulava N, Palmer GA, Mett V, et al. Plant-expressed HA as a seasonal influenza vaccine candidate. *Vaccine* 2008; 26:2930–4; PMID:18440103; <http://dx.doi.org/10.1016/j.vaccine.2008.03.045>
- Musiyuchuk K, Stephenson N, Bi H, Farrance CE, Orozovic G, Brodelius M, Brodelius P, Horsey A, Ugulava N, Shamloul AM, et al. A launch vector for the production of vaccine antigens in plants. *Influenza other Respir viruses* 2007; 1:19–25; PMID:19453476; <http://dx.doi.org/10.1111/j.1750-2659.2006.00005.x>
- Shoji Y, Bi H, Musiyuchuk K, Rhee A, Horsey A, Roy G, Green B, Shamloul M, Farrance CE, Taggart B, et al. Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. *Vaccine* 2009; 27:1087–92; PMID:19100806; <http://dx.doi.org/10.1016/j.vaccine.2008.11.108>
- Matsuoka M, Yamamoto N, Kano-Murakami Y, Tanaka Y, Ozeki Y, Hirano H, Kagawa H, Oshima M, Ohashi Y. Classification and Structural Comparison of Full-Length cDNAs for Pathogenesis-Related Proteins. *Plant Physiol* 1987; 85:942–6; PMID:16665835; <http://dx.doi.org/10.1104/pp.85.4.942>
- Green BJ, Fujiki M, Mett V, Kaczmarczyk J, Shamloul M, Musiyuchuk K, Underkoffler S, Yusibov V, Mett V. Transient protein expression in three *Pisum sativum* (green pea) varieties. *Biotechnol J* 2009; 4:230–7; PMID:19156736; <http://dx.doi.org/10.1002/biot.200800256>
- Zhu D, Saul A, Huang S, Martin LB, Miller LH, Rausch KM. Use of o-phthalaldehyde assay to determine protein contents of Alhydrogel-based vaccines. *Vaccine* 2009; 27:6054–9; PMID:19660590; <http://dx.doi.org/10.1016/j.vaccine.2009.07.067>
- Goantala J, Grover S, Goel D, Rahi A, Jayadev Magani SK, Chandra S, Bhatnagar R. A plant based protective antigen ; PA(dIV) vaccine expressed in chloroplasts demonstrates protective immunity in mice against anthrax. *Vaccine* 2011; 29:4521–33; PMID:21504775; <http://dx.doi.org/10.1016/j.vaccine.2011.03.082>
- Chen Q, Huafang L, Hurtado J, Stahnke J, Leuzinger K, Dent M. Agroinfiltration as an Effective and Scalable Strategy of Gene Delivery for Production of Pharmaceutical Proteins. *Adv. Tech Biol. Med.* 2014 1(1): 1–21
- Yusibov V, Streatfield SJ, Kushnir N. Clinical development of plant-produced recombinant pharmaceuticals: Vaccines, antibodies and beyond. *Human Vaccines* 2011; 7(3): 313–21; PMID:21346417; <http://dx.doi.org/10.4161/hv.7.3.14207>
- Elbehri A. Biopharming and the food system: examining the potential benefits and risks. *AgBioForum* 2005, 8(1):18–25
- Howard JA and Hood EE (Eds.). *Commercial Plant-Produced Recombinant Protein Products: Case Studies, Biotechnology in Agriculture and Forestry* Springer-Verlag Berlin 2014:68
- Lai H, Chen Q. Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current Good Manufacturing Practice regulations. *Plant Cell Rep.* 2012 31:573–584; PMID:22134876; <http://dx.doi.org/10.1007/s00299-011-1196-6>
- Fischer R, Schillberg S, Hellwig S, Twyman RM, Drosard J. GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnology Advances* 2012 30: 434–439; PMID:21856403; <http://dx.doi.org/10.1016/j.biotechadv.2011.08.007>
- Wirz H, Sauer-Budge AF, Briggs J, Sharpe A, Shu S, Sharon A. Automated Production of Plant-Based Vaccines and Pharmaceuticals. *J. Lab. Autom.* 2012 17(6) 449–457; PMID:23015521; <http://dx.doi.org/10.1177/2211068212460037>
- FDA/USDA Draft Guidance. *Biologics, and medical devices derived from bioengineered plants for use in humans and animals.* FDA, Rockville MD; 2002; 2–20; <http://www.fda.gov/downloads/AnimalVetinary/GuidanceComplianceEnforcement/Guidanceforindustry/UCM055424.pdf>