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Expression of the *Necator americanus* hookworm larval antigen *Na*-ASP-2 in *Pichia pastoris* and purification of the recombinant protein for use in human clinical trials

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Abstract

The ASP-2 protein secreted by infective larvae of the human hookworm, *Necator americanus*, is under development as a recombinant vaccine. Recombinant *Na*-ASP-2 was expressed in *Pichia pastoris*, and the purified protein was characterized. At the 60 L scale, the 21.3 kDa recombinant protein was produced at a yield of 0.4 g/L. When formulated with Alhydrogel® and injected into rats to determine immunological potency, three 50 μg doses of the formulated recombinant protein elicited geometric mean antibody titers up to 1:234,881. Rat anti-*Na*-ASP-2 antibody recognized larval-derived ASP-2 and also inhibited larval migration through skin in vitro. The processes developed and tested for the high yield production of recombinant *Na*-ASP-2 provide a foundation for clinical vaccine development.

Keywords: Hookworm; Vaccine; *Necator americanus*; *Na*-ASP-2

1. Introduction

Human hookworm infection caused by *Necator americanus* or *Ancylostoma duodenale* is a major cause of iron deficiency anemia and protein malnutrition in the developing world and a leading cause of parasitic disease burden [1,2]. An estimated 740 million people are infected in areas of rural poverty in the tropics and subtropics resulting in up to 65,000 deaths and 22 million disability-adjusted life years [3,4]. The major approach to hookworm control relies on reducing morbidity through the frequent and periodic use of benzimidazole anthelminthic (BZAs) drugs, which can temporarily remove adult parasites in the human gastrointestinal tract [4,5]. Such treatment for school-aged children leads to improvements in health and educational achievement [4]. However, there are reasons why exclusive reliance on BZAs for controlling hookworm infection may not be effective. Among them, post-treatment hookworm re-infection rates are high in areas of high transmission [6], and the efficacy of BZAs can diminish

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with increasing and frequent use [7]. It has been further suggested that the emergence of BZA drug resistance is possible [7]. School-based programs are also not expected to interrupt hookworm transmission [8].

In order to develop new tools for the control of hookworm infection, studies were undertaken to develop recombinant hookworm vaccines. Based on previous success in the development of canine hookworm vaccines from third-stage infective larvae (L3) that were attenuated by X-irradiation [9], our antigen discovery efforts focused on the identification and isolation of critical L3-secreted molecules that mediate protective immunity [10]. These included a novel class of cysteine-rich secretory proteins known as the *Ancylostoma* secreted proteins or ASPs [10–14]. The ASPs belong to the pathogenesis-related (PR) protein superfamily whose members are found in a wide variety of animals, plants and fungi where they are typically produced in response to stress or injury, including invasion by pathogens [15]. Although the function of the hookworm larval ASPs is not known, the observation that they are released by L3 upon receiving host-specific stimuli suggests that these proteins are released during host entry and function in the transition from the environment to a parasitic existence [13].

ASP-2 was selected for evaluation as a recombinant vaccine candidate based on human immunoepidemiologic studies and laboratory animal vaccine trials [16–19]. These data led to the selection of ASP-2 from the human hookworm *N. americanus* for further development. Here we describe the cloning of the *Na-asp-2* gene and the scale-up expression of the purified recombinant *Na*-ASP-2 protein in the yeast, *Pichia pastoris*. We further describe the immunological potency of *Na*-ASP-2 and the cross-reactivity of antibody against the recombinant protein with the native, parasite-derived protein. The studies here also confirm the importance of *Na*-ASP-2 in hookworm tissue migration. These data lay the foundation for the clinical development of the *Na*-ASP-2 Hookworm Vaccine.

2. Materials and methods

2.1. Molecular cloning of Na-asp-2 cDNA and transformation into Pichia pastoris

A cDNA library was constructed with mRNA from L3 of a Chinese strain of *N. americanus* [20], and probed with a heterologous cDNA fragment of *Ac-asp-2* cloned from the dog hookworm *Ancylostoma caninum* [12]. The *Ac-asp-2* pBluescript plasmid was cut with *Xho*I and *Bam*HI, releasing a 739 bp fragment (28–767 bp). The fragment was gel purified (Qiagen) and randomly labeled with α-^{32}[P]-dCTP using a Rediprime labeling kit (Amersham). Approximately 5×10^5 plaques of *N. americanus* cDNA library were screened as previously described [20]. The positive clones were excised and subjected to secondary screening with the same reagents to isolate single positive colonies. The positive clones from secondary screening were excised in vivo as pBluescript phagemids according to the manufacturer's instruction (Stratagene). Phagemid DNA was extracted using the alkaline lysis method (Qiagen), and both strands were sequenced using vector primers, T3 and T7.

The complete 5′ end of *Na-asp-2* cDNA was isolated from first strand cDNA of *N. americanus* L3 by a modified RNA ligase-mediated rapid amplification technique (GeneRacer; Invitrogen) as described previously [21]. The *Na-asp-2* gene specific primers used for isolating the 5′ end were Na-ASP2-R2 (TGT CTA GAT CAA GCA CTG CAG AGT CCC TTC TC) and Na-ASP2-R1 (TGT CTA GAG CAC TGC AGA GTC CCT TCT C). The PCR products were ligated into the T-ended vector pGEM-T (Promega) according to the manufacturer's instructions. Recombinant plasmids containing the correct insert were extracted using a Qiaprep Spin Miniprep kit (Qiagen) and submitted for dideoxy terminator cycle sequencing. DNA and predicted protein sequences were analyzed using ESEE version 3.1 [22]. Amino acid sequences were analyzed for putative signal peptides using signalP (http://www.cbs.dtu.dk/services/SignalP-2.0). Subsequently, an additional round of screening of the *N. americanus*-L3 cDNA library was performed to confirm that there were no other *Ac-asp-2* cDNA orthologues.

The entire coding sequence minus the N-terminal signal peptide of the *Na-asp-2* gene was amplified by PCR from the first strand cDNA of *N. americanus* L3 with *Na-asp-2* gene-specific primers and subcloned into the *Pichia* expression vector, pPICZαA (Invitrogen, CA) as described previously [15]. The recombinant plasmids were linearized with *Sac*I digestion and transformed into *P. pastoris* X33 strain as described previously [15–17]. Seed stocks were prepared from a colony of the transformed *P. pastoris*.

2.2. Expression of Na-ASP-2 in Pichia pastoris

Expression of *Na*-ASP-2 in *P. pastoris* was conducted at the 60 L scale and at the 10 L scale.

For 60 L scale expression, four 2.5 L Tunair shake flasks (Shelton Scientific, CT), each containing 1 L of Buffered Minimal Glycerol (BMG) medium (1.34% Yeast Nitrogen Base, 0.00004% (w/v) d-biotin, 1% (w/v) glycerol and 100 mM potassium phosphate, pH 6.0) were inoculated with 2 mL of the *P. pastoris* seed stock. The flasks were incubated at $30 \pm 2\,^{\circ}$C for 24–25 h to a final OD_{600} of 5–15. Approximately 3 L of this culture was used to inoculate 30 L of heat-sterilized basal salt media (BSM) containing 3.5 mL/L of a filter-sterilized trace element (PTM4) solution [16,23]. Fermentation was conducted in a BioFlo5000 Mobile Pilot Plant (New Brunswick Scientific Co. Inc.). The growth of *P. pastoris* at the 60 L scale was divided into two phases: a fed-batch glycerol phase and a methanol induction phase. The pH of the BSM was adjusted to and maintained at 5.0 with 14% ammonium hydroxide feed. The agitation speed was set at 550 rpm and the dissolved oxygen maintained at 30% throughout the fermentation. At approximately 18 h into the glycerol phase