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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

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ABSTRACT

The enzyme *Necator americanus* glutathione S-transferase 1 (*Na*-GST-1) belongs to a unique Nu class of GSTs and is a lead candidate antigen in a bivalent human hookworm vaccine. Here we describe the expression of *Na*-GST-1 in the yeast *Pichia pastoris* at the 20 L manufacturing scale and its purification process performed by three chromatographic steps, comprised of a Q Sepharose XL anion exchange column, followed by a Butyl Sepharose HP hydrophobic affinity column and a Superdex 75 size-exclusion column. Approximately 1.5 g of recombinant protein was recovered at an overall process yield of 51%, with a purity grade of 98% and the absence of detectable host cell protein. By mass spectrometry the recombinant protein exhibits a mass of 23,676 Da, which closely matches the predicted molecular mass of the protein. The expression and purification methods described here are suitable for further scale-up product development and for its use to design formulation processes suitable to generate a vaccine for clinical testing.

Introduction

Human hookworm infection is a major cause of iron deficiency anemia and protein malnutrition in the world's low- and middle-income countries in Africa, Asia, and Latin America [1]. An estimated 600 million people are infected with hookworms worldwide, with most of the cases caused by *Necator americanus* [2]. Despite the widespread availability of benzimidazole anthelminthic drugs, hookworm infection remains a significant global health threat due to the high rates of mebendazole drug failure, and rapid post-treatment re-infection with albendazole (reviewed in Ref. [3]). Hence there is an urgent need for new control tools to combat hookworm infection including anthelminthic vaccine. A human hookworm vaccine is under development by the Sabin Vaccine Institute Product Development Partnership (Sabin PDP) [3]. The vaccine is comprised of two recombinant hookworm antigens, known as *N. americanus* aspartic protease 1 (*Na*-APR-1) and *N. americanus* glutathione S-transferase 1 (*Na*-GST-1),[2] each of which is a macromolecule involved in parasite blood feeding [3]. *Na*-APR-1 is an aspartic protease that degrades hemoglobin, but which has been modified through site directed mutagenesis to inactivate its hydrolytic function and yet retain its overall confirmation [3,4]. *Na*-GST-1 is a specially adapted Nu-class glutathione S-transferase that forms a heme- and hematin-binding pocket during homodimer formation [5–8]. *Na*-GST-1 is believed to function in heme detoxification during parasite blood feeding [5–8]. In preclinical testing recombinant *Na*-GST-1 expressed in yeast, as well as its orthologous enzyme from the dog hookworm *Ancylostoma caninum* (*Ac*-GST-1), elicit high levels of protective immunity against hookworm larval challenge infections, as evidenced by reductions in the numbers of adult hookworms relative to negative controls [5–8]. Here we describe the manufacturing of a 20 L scale fermentation process for the expression of *Na*-GST-1 in the yeast *Pichia pastoris* and its subsequent purification through three chromatographic steps. In addition, we provide in process characterization data to confirm the overall recovery (yields) and purity of the recombinant *Na*-GST-1 protein. The expression and purification

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[2] *Abbreviations used:* *Na*-APR-1, *N. americanus* aspartic protease 1; *Na*-GST-1, *N. americanus* glutathione S-transferase 1; cGMP, current good manufacturing practices; BMG, buffer media with glycerol; BSM, basal salt media; WCW, wet cell weight; CFS, concentrated fermentation supernatant.

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reported here were considered suitable for a pilot manufacture of a recombinant vaccine under current good manufacturing practices (cGMP).

Methods

Expression of Na-GST-1

The cloning of the gene encoding *Na*-GST-1 into *P. pastoris* has been reported previously [8]. A schematic overview of the processes used for the 20 L fermentor expression of *Na*-GST-1 is shown in Fig. 1A. Briefly, five vials of the working cell bank were grown to obtain a sufficient cell density for inoculation of the production fermentor. This step is performed in four 2-L buffered shake flasks containing 0.8 L of sterile buffer media with glycerol (BMG). Three of the shake flasks were harvested, while the fourth served as an optical density proxy and incubated at 30 ± 1 °C with agitation at 250 ± 10 rpm for approximately 27–30 h until the OD600 of the culture is 10.0 ± 4.0 was reached. Upon reaching the acceptable OD, a 20 L fermentor was inoculated containing 10 L of basal salt media (BSM) containing 3.5 ml/L of a 0.02% (D)-biotin solution. Fermentation was conducted in a 20 L Bioengineering fermentor model NLF-22 (Bioengineering, Switzerland). The pH of the BSM was adjusted to and maintained at 5.0 with 14% ammonium hydroxide feed. Cells were grown at 30 ± 1 °C in 30% dissolved oxygen and at an agitation speed of 450 rpm. Approximately 18 h into the glycerol phase and after a sharp increase in the percentage of dissolved oxygen (indicating depletion of glycerol), 50% (v/v) glycerol was introduced into the cell culture medium at a set flow rate of 15 g/L/h, for 6 h. The pH of the culture was increased linearly from 5.0 to 6.0 by adding 14% ammonium hydroxide and the temperature linearly decreased from 30 to 26 °C over a 2-hour period before the completion of the fed-batch glycerol phase. Excessive foaming was controlled with 10% (v/v) antifoam KFO673 (KABO Chemicals Inc.) in deionized water. The agitation speed was increased from 450 to 700 rpm. The methanol induction phase was initiated at a wet cell weight (WCW) of approximately 200 g/L and increased from 1.5 to 11.0 ml/L/h over an 8-hour period. Methanol induction was continued for another 57 h by pumping 100% methanol at a flow rate of 11 ml/L of BSM/h until harvest at a WCW of approximately 463 g/L. Centrifugation [@7000 rpm and 4°C for 30 min using Avanti J-26 XPI and JLA 8.1000 rotor (Beckman)] was used to remove the cells and cellular debris and to recover the supernatant (~17 L) containing the recombinant *Na*-GST-1. This supernatant was filtered using a 0.8 and 0.2 μm sterile depth filter and then concentrated to 4 L by using an ultrafiltration unit consisting of a Masterflex Pump and a 3 kDa hollow fiber cartridge (UFP-3C-55-GE Healthcare). Concentrated fermentation supernatant (CFS) was washed using ultrafiltration with additional volume of 20 mM Tris HCl buffer, pH 8.5 to decrease the conductivity ~5.0 mS and increase the pH ~8.5. Finally, this CFS

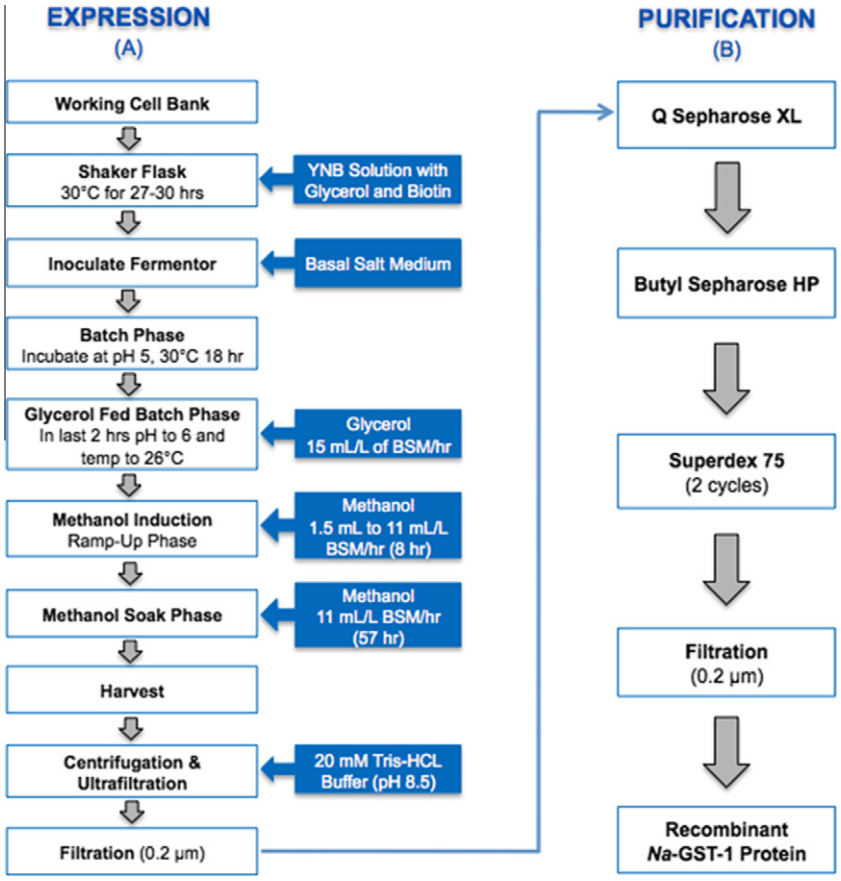

Fig. 1. Flow diagram of the steps used to express and purify recombinant *Na*-GST-1. (A) Shows the expression of *Na*-GST-1 in *P. pastoris* beginning in the shaker flask using the research cell bank of pPicZalpha-A containing the DNA sequence encoding *Na*-GST-1. Following inoculation of a 10 L fermenter with 5 L basal salt medium (BSM) the cells were grown in a glycerol batch phase followed by induction of protein expression with methanol. After harvest, microfiltration and ultrafiltration prepare the target antigen for subsequent purification. (B) Shows the downstream purification utilized including a capture chromatography step via ion exchange chromatography on Q XL media. A butyl HP step served as an additional polishing step followed by removal of high molecular weight host cell proteins (HCPs) via Superdex 75 size exclusion chromatography. The final *Na*-GST-1 recombinant protein was sterile filtered and subsequently stored.