Vaccine 31 (2013) 4714–4719

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A novel method for purification of Vi capsular polysaccharide produced by *Salmonella enterica* subspecies *enterica* serovar Typhi

Sudeep Kothari[a,c], Neha Kothari[a], Jeong-Ah Kim[a], Eugene Lee[a], Yeon Kyung Yoon[a], So Jung An[a], Christopher Jones[b], Woo Seok Choe[c], Rodney Carbis[a,*]

[a] *Vaccine Development Section, International Vaccine Institute, SNU Research Park, San 4-8, Nakseongdae-dong, Gwanak-gu 151-919, Seoul, Republic of Korea*
[b] *National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK*
[c] *School of Chemical Engineering, Sungkyungkwan University, Suwon 440-746, Republic of Korea*

ARTICLE INFO

Article history:
Received 4 June 2013
Received in revised form 9 August 2013
Accepted 13 August 2013
Available online 28 August 2013

Keywords:
Salmonella Typhi
Vi polysaccharide
Polysaccharide purification
Typhoid vaccine

ABSTRACT

Vi capsular polysaccharide is the major component of Vi polysaccharide typhoid vaccines. Vi is synthesized during growth of *Salmonella enterica* subspecies *enterica* serovar Typhi and is released into the fermentation broth in large quantities. Along with the Vi considerable amounts of impurities consisting of bacterial protein, nucleic acid and lipopolysaccharide (LPS) as well as media components contaminate the fermentation broth. A purification method based on selective precipitation of Vi using the cationic detergent cetavlon was developed to separate impurities from Vi. A novel method for handling the Vi precipitate using 0.2 μm sterilizing grade filters to trap and wash the Vi and then, after re-solubilization, allow the Vi to pass through the filter was developed. Cetavlon selectively precipitates Vi and is the major purification step in the process, however, the conditions must be carefully controlled otherwise LPS will co-precipitate in large quantities. Various diafiltration steps help to remove contaminating protein, nucleic acid and fermentation media components as well as chemicals added during the process to induce precipitation of either Vi or contaminants. The final yield of purified Vi was approximately 45% and the bulk concentrate complied with the specifications defined in the WHO recommendations for Vi polysaccharide vaccine. Analysis of the Vi by size exclusion chromatography revealed a uniform peak with a narrow size distribution. The Nuclear Magnetic Resonance spectrum was similar to Vi produced by other methods. The method developed produces large quantities of Vi using low cost production methods translating into Vi based vaccines that can be produced at affordable prices for use in developing countries.

1. Introduction

Typhoid fever caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi) continues to present a significant disease burden predominantly in the poorest communities in developing countries where sanitation is inadequate and spread of disease a constant problem. WHO estimates the global incidence of typhoid fever to be 21 million cases, with a fatality rate ranging between 1% and 4% [1]. The Vi capsular polysaccharide synthesized by *S.* Typhi has been shown to be a protective antigen against typhoid fever [2]. The capsular polysaccharide of Salmonella Typhi is a linear homopolymer of α1,4-N-acetylgalactosaminouronic acid, 60–90% O acetylated at the C-3 position [3]. Vi based vaccines have been available for many years but regrettably persons living in communities where typhoid fever is endemic remain largely unvaccinated [4]. One reason for the lack of vaccine uptake in endemic settings is that people living in these communities do not have the capacity to pay and there is limited donor funding for typhoid vaccination. To address the issues of vaccine costs optimization of the growth of *S.* Typhi and production of Vi was performed which resulted in significant increases in yield of Vi [5]. This paper describes the development of a purification method aimed to maximize recovery of Vi while removing impurities to acceptable levels. It is intended to make the technology available to developing country vaccine manufacturers, with that in mind the process has to be scalable and compatible with cGMP (current Good Manufacturing Practice) requirements [6]. To address the issue of scale up for manufacture only equipment that has a large scale equivalent and has demonstrated scalability was used in the development of this Vi purification method. From a cGMP perspective the use of chemicals such as phenol were excluded, the use of equipment that required extensive cleaning and cleaning validation was avoided,

* Corresponding author. Tel.: +82 2 881 1169; fax: +82 2 881 1239.
E-mail address: rcarbis@ivi.int (R. Carbis).

0264-410X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.08.037

preferring where possible to employ single-use technologies [7]. In keeping with the ideal of producing vaccine at an affordable price it was important to minimize the need for capital equipment such as centrifuges and large scale chromatography.

Working within these constraints a reliable purification process with demonstrated consistency was developed. The importance of process control especially during the cetavlon precipitation of the Vi is highlighted and a novel methodology developed for handling the precipitate is introduced.

2. Materials and methods

2.1. Bacterial strain and cultivation in a bioreactor

S. Typhi clinical isolate C6524 was obtained from National Institute of Cholera and Enteric Diseases (NICED) Kolkata India. This isolate was used to prepare a master seed lot and was shown to be a high yielding isolate of Vi. C6524 was cultivated in a bioreactor using the optimized growth conditions described earlier [5] with fermentation conditions as follows: base media contained 10 g/L yeast extract (Oxoid Ltd.), 5 g/L casamino acid (Becton Dickinson and Co.), 3 g/L glucose (Junsei chemical Co.), 2.5 g/L $MgSO_4{\cdot}7H_2O$ (Junsei Chemical Co.), 4.1 g/L $NaH_2PO_4{\cdot}H_2O$ (USB Corporation) and 18.8 g/L $Na_2HPO_4{\cdot}7H_2O$ (USB Corporation), the feeding solution contained 100 g/L yeast extract, 100 g/L casamino acid and 300 g/L glucose. All media components were sterilized by filtration through a Sartopore 300 (Sartorius-Stedim) 0.2 μm filter which was placed in line on the fermentor and sterilized in situ during sterilization of the fermentor vessel. The pH was controlled at 7.2, dissolved oxygen at 35% air saturation and the temperature maintained at 32 °C. Feeding was started when the glucose concentration dropped to 1 g/L: glucose was monitored at 60 min intervals and the pump controlling the feed solution was adjusted to maintain the glucose concentration at about 1 g/L glucose. Once stationary phase was reached feeding was discontinued and stationary phase allowed to continue for a total of 2 h. At the end of the 2 h stationary phase fermentation was stopped and the cells inactivated by addition of formaldehyde to a final concentration of 0.15%. Fermentation broth was harvested and stored at 4 °C overnight. The starting volume of media was 2.5 l plus 250 ml of seed culture and at the end of feeding the volume of culture broth was 3.4 l.

2.2. Clarification of crude Vi

The fermentation broth was clarified using 0.45 μm Hydrosart microfiltration cassettes (Sartorius-Stedim) installed in a Sartocon alpha holding device (Sartorius-Stedim). The bacterial cells were concentrated approximately 7 fold ensuring that the inlet pressure did not exceed 2.0 bar, the retentate line was not restricted. At the conclusion of concentration the cells were diafiltered against 10 volume changes of 1 M NaCl maintaining a constant retentate volume to ensure maximal recovery of Vi in the permeate. The permeate collected during concentration was pooled with the permeate collected during diafiltration. The permeate pool was concentrated 16 fold and diafiltered against 10 volume changes of purified water using a 30 kD Hydrosart cassette (Sartorius-Stedim) installed in the Sartocon alpha. During concentration/diafiltration the inlet pressure was maintained at 1.0 bar and the retentate was not restricted. The concentrate was then diluted with purified water such that the final volume was approximately double that harvested from the bioreactor.

2.3. Purification of Vi

The crude Vi and a 10% cetavlon (Hexadecyl trimethylammonium bromide – Sigma) solution were heated to 30 °C. While stirring, the 10% cetavlon was slowly added to the crude Vi so that the final concentration of cetavlon was 0.5% then the mixture was held at 30 °C for 2 h. The mixture was pumped through a 0.2 μm Sartopore 2 (Sartorius-Stedim) capsule filter and the filtrate recirculated until the filtrate became clear. Filtration was continued until all of the liquid has passed through the membrane. The precipitate trapped on the membrane was washed by pumping 20% ethanol (Duksan) in 50 mM sodium acetate (UBS) pH 6.0 through the filter at a rate of 1 l per 3 l of original fermentation broth. The Vi was then re-dissolved by slowly pumping 60% ethanol (in 50 mM sodium acetate pH 6.0) through the filter at a rate of 2 l per 3 l of original fermentation broth. 5 M NaCl was added to the filtrate to give a final concentration of 1 M NaCl then the Vi was re-precipitated by adding ethanol to bring the final concentration up to 75%. The precipitate was allowed to form and settle to the bottom of the container by incubating the mixture overnight at 4 °C. Without disturbing the precipitate the supernatant was pumped through a Sartopore 2 300, the precipitate was then washed with ethanol (300 ml per 3 l of original fermentation broth) then allowed to resettle for 1 h, the supernatant was then pumped through the filter. The precipitate was re-dissolved in water (at a rate of 300 ml per 3 l of original fermentation broth) and pumped through the filter. 40% (w/v) $(NH_4)_2SO_4$ was then added to the solubilized Vi to give a final concentration of 10% (w/v) and stirred for 2 h at room temperature. The precipitated impurities were then removed by pumping the mixture through a Sartopore 2 300. The Vi in the filtrate was concentrated 10 fold using a 100 kD Hydrosart cassette installed in a Sartocon alpha, after concentration the retentate was constant volume diafiltered against 20 volume changes of purified water. During concentration/diafiltration the inlet pressure was maintained at 1.0 bar and the retentate was not restricted. The Vi concentrate was sterile filtered through a Sartopore 2 300.

2.4. Quality control and characterization assays

The Vi polysaccharide was assayed for *O*-acetyl content by Hestrin assay [8], for protein by Lowry assay [9] and for nucleic acid by ultraviolet sprectroscopy [10]. Endotoxin levels were measured by Limulus Amebocyte Lysate (LAL) kinetic-turbidimetric assay as described by the supplier (Cambrix Cat. No. N588). Molecular size was determined by size exclusion chromatography using Sepharose CL4B (GE Healthcare) and the percentage of Vi eluting before a K_D of 0.25 determined by Hestrin [10]. Additional size exclusion chromatography was performed using Sephacryl S-1000 (GE Healthcare) to more accurately define the size distribution of the Vi. Dry weight of the consistency batches was determined by freeze drying 100 ml of each batch for three days in a CnH Lab Mast freeze dryer then measuring the weight of the dried powder. The Vi content of in-process and final bulk concentrate was measured using ELISA. Briefly the method was as follows: ELISA plates (Maxisorp, Nunc) were coated with Vi at 1 μg/ml. Two fold dilutions of standard Vi and samples were prepared in separate plates. An equal volume of rabbit anti-Vi serum (prepared by AbFRONTIER (Korea) using Vi-Diphtheria Toxoid conjugate prepared at IVI) was added to each Vi dilution and incubated for 1 h at room temperature (RT). The Vi/anti Vi mixtures were transferred to corresponding wells in the Vi coated plates and incubated at RT for 1 h. Plates were then washed and goat anti-rabbit alkaline phosphatase conjugate (Jackson Immuno Research Laboratories) added. After 1 h at 37 °C plates were washed and substrate (4-Nitrophenyl phosphate (Sigma)) added. The plates were read at 405 nm using an ELISA reader (Ultramark; Biorad).

NMR spectra of the native and de-O-acetylated polysaccharides were collected on an Inova 500 spectrometer (Varian Associates, Abingdon) equipped with a 5 mm inverse detection heteronuclear