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MOLECULAR CHARACTERIZATION AND OPTIMIZATION OF VI-CAPSULAR POLYSACCHARIDE OF *SALMONELLA TYPHI* TY6S PRODUCTION IN BIOREACTOR

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ABSTRACT

The role of Vi-capsular polysaccharide (Vi-CPS) in human immunity against infection caused by *Salmonella typhi* is well known. The downstream process of purification generally causes depolymerization of Vi-CPS to a nonimmunogenic low molecular weight form. In the present study, a standard strain of *Sal. typhi* Ty6s was grown under submerge cultural conditions in a pilot-plant scale of 90 liter fermentor. At the late exponential growth phase, crude polysaccharide was obtained from fermentation broth by detergent-phenol extraction method and purified by ultracentrifuge differentiation technique. Analytical data reveals that by optimization of fermentation parameters, not only was the yield of production increased from 1.6 mg/L to 4.9 mg/L, but also the polysaccharide retained its native molecular stability and immunogenicity. Therefore, purified Vi-CPS can be regarded as a reliable immunogen to control typhoid fever in man.

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Keywords: *Salmonella typhi*, Vi-CPS, Polysaccharide vaccine.

INTRODUCTION

Typhoid fever still remains as one of the serious health problems in developing countries[3,10] with 12.5 million cases annually (except China).[3,23] In fact the final goal to control this infection is vaccination.[2, 4, 18, 20, 21-23] Classical TAB-typhoid vaccine[4] and oral *Sal. typhi Ty21a* vaccine[22] offer good protection in man[2,22] but each of these suffers from some drawbacks as an ideal vaccine[2,4,18,22] which makes this field open for further investigations.

Recently, it has been shown in two controlled field trials that Vi-CPS sub-unit vaccine could prevent typhoid fever. A single intra-muscular injection of this vaccine conferred about 65%[12] to 72%[1] protection in South Africa and Nepal, respectively.

The production rate of Vi-CPS in laboratory scale, under stationary cultural growth conditions, is low.[5] Scaling

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up the rate of production to a large scale is technically difficult and causes depolymerization of Vi-CPS. Besides, downstream processes are either patented or kept secret in multi-national companies marketting vaccines.

Our investigations were aimed at overcoming this problem by adaptation of *Sal. typhi* Ty6s R-mutant in submerged growth cultural conditions in five liters of bioreactor and optimized cultural conditions, then scaling the production rate up to ninety liters while retaining the native molecular and immunological identity of Vi-CPS after extraction and purification.

Analysis of obtained experimental data revealed that after adaptation to our procedure, Vi-CPS retains its native molecular status even in large scale production rates. Therefore Vi-CPS can be regarded as a safe and reliable immunogen for mass vaccination to control typhoid fever in man.

Further immunological investigations in volunteers are necessary to prove this claim in Iran.

MATERIALS AND METHODS

Bacterial strains

Vi-CPS was produced from a standard rough strain of *Sal. typhi* Ty6s (CSBPI-B191) which is rich in Vi-polysaccharide. This strain was procured from Collection of Standard Bacteria, Department of Bacterial Vaccines, Pasteur Institute of Iran. Hyperimmune Vi-antiserum was prepared from *Citrobacter ballerup* (CSBPI-A124)

Culture

Sal. typhi Ty6s was grown on 90 liters of modified Frantz medium[6] containing 5 g/L yeast extract-dialysate, 5 g/L glucose in classical batch and 20 g/L glucose in fed-batch processes in a NOVO-Pal Jas Bilthoven unit bioreactor. Fermentation parameters were adjusted at 36±1°C, pH=7.6, final speed of rotation 450 rpm and air flow 5 L/h.

Purification of Vi-CPS

At the late exponential growth phase (14 h), cells were harvested from fermentation broth by centrifugation at 4000 rpm at 4°C. The supernatant was treated with 1g/L of cetavlon (Fluka Chemical, AG.) with continuous shaking at 4°C for 30 minutes. The precipitate was collected by centrifugation at 4000 rpm at 4°C for 30 minutes. Crude Vi-CPS was obtained according to the method of Tacket et al.[20] The Vi-CPS was further purified by ultra-centrifuge differentiation technique[7] using a Beckman Instruments Inc. LM 80 ultracentrifuge.

Purified and filter sterilized Vi-CPS was washed twice with alcohol, acetone and diethyl ether, then dried in a p205 desiccator at 37°C. Dried Vi-CPS was dispensed in 50 mL vials and kept at -20°C until used.

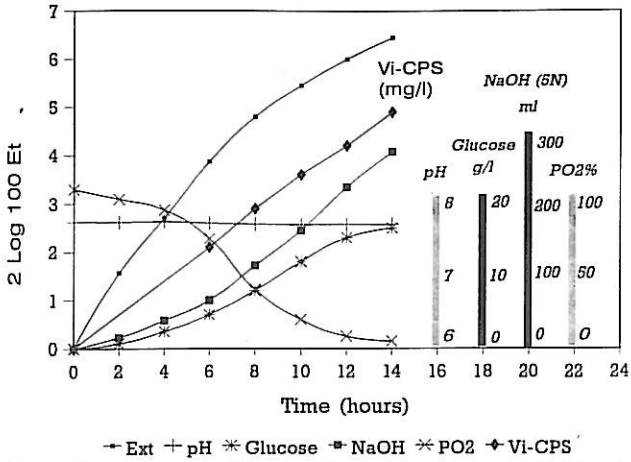

Fig. 1. Growth curve of *Salmonella typhi* in fermentor (fed batch culture).

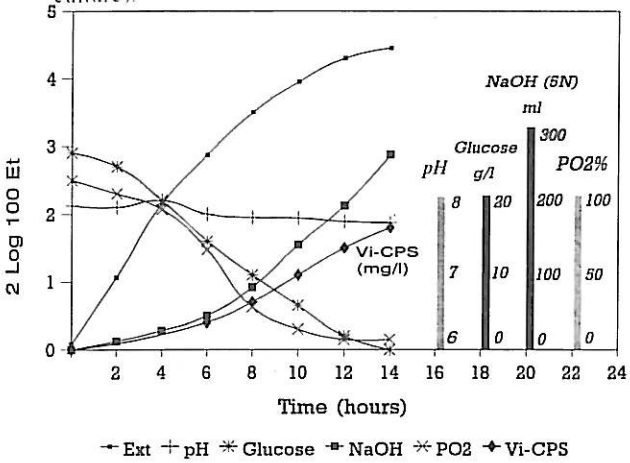

Fig. 2. Growth curve of *Salmonella typhi* in fermentor (batch culture).

Moisture content

The moisture content of the purified Vi-CPS was determined by drying under vacuum and P205 at 37°C until a constant weight was reached.

O-Acetyl content

O-acetyl content of purified Vi-CPS was determined according to Hestrin,[8] using acetylcholine chloride as a reference.

Nucleic acid and protein contamination

Nucleic acid was determined by U.V. spectroscopy,[25] and protein was determined by Lowry's method[14] using bovine serum albumin (BSA) as a reference.

Molecular size

The molecular size of purified Vi-CPS was estimated by gel filtration chromatography column (1.5×85) using sepharose 4B-CL.[17, 25] The polysaccharide was eluted by 0.2M sodium choride containing 2 mM Na_3N. The content of Vi-CPS in each fraction tube was measured by the

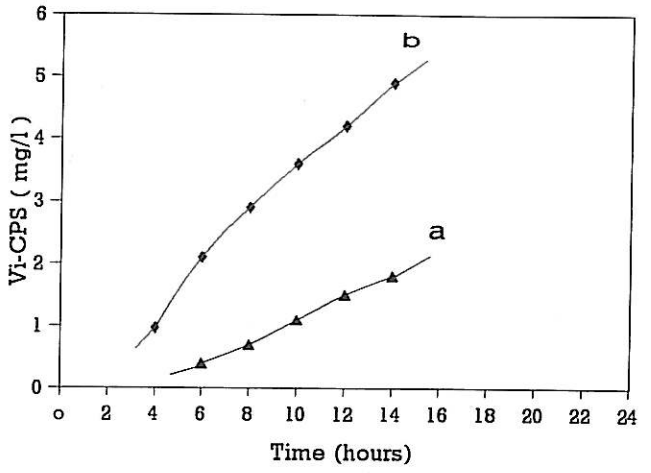

Fig. 3. Comparison of production rate of Vi-CPS **a)** before and **b)** after optimization of fermentation parameters.

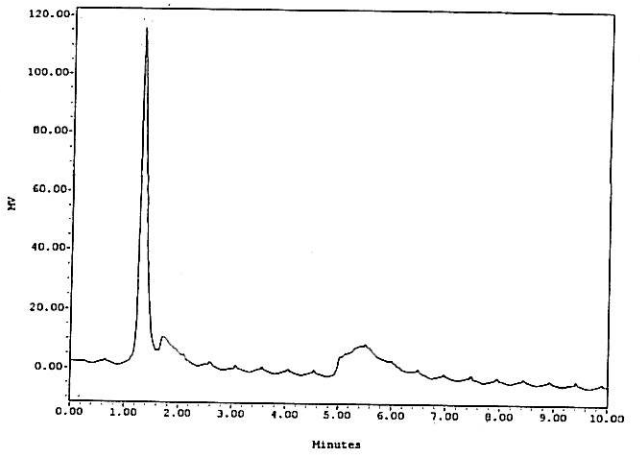

Fig. 4. HPLC profile of purified Vi-CPS, passed through a reverse phase column (p18, Delta pack, 5 micron, 100 Å, size 3.9x150mm) and R-410 differential refractometric detector.

Mv= Maximum velocity.

method of Hestrin.[8]

Sterility test

The purified Vi-CPS was tested for bacterial and mycotic sterility according to the requirements given in the revised WHO Requirements for Biological Substances.[26]

Abnormal toxicity test

The purified Vi-CPS was tested for abnormal toxicity by intraperitoneal injection of 50 µg purified Vi-CPS into five mice (weighing 17-22g) and 250 µg purified Vi-CPS into two guinea-pigs (weighing 250-350 g). The test was considered satisfactory if the animals would survive for at least seven days without weight loss.

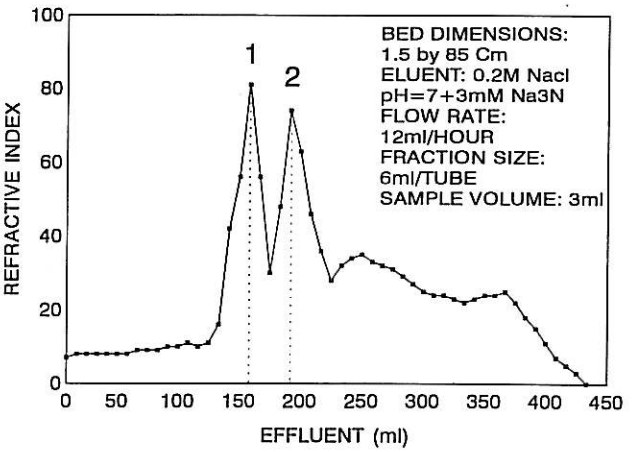

Fig. 5. Elution profile for *Salmonella typhi* Ty6s Vi-CPS on sepharose 4B-CL column.

Pyrogenicity test

The purified Vi-CPS was tested for pyrogenic activity by intravenous injection into rabbits (New Zealand White rabbits weighing 2 to 2.5 kg each). Three healthy rabbits were used in each test. The polysaccharide was reconstituted in special diluent. Further dilution was done in pyrogen-free physiological saline. Each rabbit received 0.025 micrograms of purified Vi-CPS per kg of rabbit weight.[19]

Immunogenicity studies

In this study, five New Zealand White rabbits (2 to 2.5 kg each) were used. Each rabbit received 50 micrograms of purified Vi-CPS emulsified in 50%(vol/vol) complete Freund's adjuvant intramuscularly. Bleeding for serological analysis was performed at days 0, 15, 30, 45, 180, and 360. The titer of serum against Vi-CPS of each sample was determined by passive hemagglutination (PHA) technique.[13,15]

Identity test

The identity of the Vi-CPS was estimated by Ouchterlony gel double diffusion technique[16] using hyperimmune serum prepared from multiple injections of a standard culture of *Citrobacter ballerup* Vi+ (CSBPI-A124). The immunoglobulin was purified and concentrated against 45% saturated ammonium sulphate.

High pressure liquid chromatography (HPLC)

10 µg of Vi-CPS was injected to a HPLC Waters-4000, Reverse phase column (P18, Delta pack, 5 micron, 100 Å, size 3.9 ×150 mm) and R-410 differential refractometric detector. Diluent was 80% methanol with flow rate of one mL/min.

Table I. Yield of Vi-CPS production before and after optimization*(mg/L).

	Time (hour)							
	0	2	4	6	8	10	12	14
Before opt.	-	-	0	0.4	0.7	1.1	1.5	1.8
After opt.	-	-	0.97	2.1	2.9	3.6	4.2	4.9

* Increasing yeast extract from 3g/L to 5 g/L giving glucose in fed-batch process in fermentation broth and extracting Vi-CPS from live culture.

Table II. Determination of titer of pooled anti-Vi sera by passive hemagglutination.

	Antigen dilution					
Antigen	**0**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**
Vi-CPS*	+	+	+	+	+	+
Vi-CPS**	+	+	+	+	+	+
N. meningitidis A polysaccharide	-	-	-	-	-	-

* Before optimization
** After optimization
+Positive hemagglutination
- Negative hemagglutination

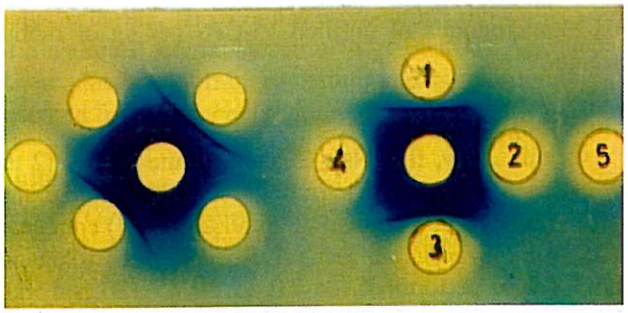

Fig. 6. Immunodiffusion analysis of native purified Vi-CPS. Wells 1, 2 and 3, purified Vi-CPS; well 4, *N. meningitidis* A polysaccharide as antigen control. Well 5, monospecific anti-LPS antiserum. *Sal. typhi* as negative control. Center well, monospecific purified Vi-antiserum.

RESULTS

The biochemical and some of the immunological properties of purified Vi-CPS prepared in a semi-industrial scale of 90 liter bioreactor were studied by different methods.

Sal. typhi Ty6s was grown under controlled-submerged cultural conditions and Vi-CPS was extracted from fermentation broth. The product was highly purified containing more than 4.7 mMol O-acetyl and less than 10 mg protein and 20 mg nucleic acid per gram polysaccharide as contaminants. Moisture content was less than 2.5% of dry polysaccharide weight.

As observed in Table I and Figs. 1, 2 and 3, after optimizing the fermentation parameters, the yield of production was increased by 3.1 mg/L.

HPLC

To determine the purity of the product and apparent molecular size, high pressure liquid chromatography was performed and only one peak with a retention time of one minute and 10 seconds was observed (Fig. 4).

Chromatography

As observed in Fig. 5, the Vi-CPS was eluted as a high molecular weight aggregate form with two major adjacent peaks at tubes No. 25-29 and 30-40, respectively.

Gel-Precipitation reaction

This method was employed to detect the identity of the product. Fig. 6 illustrates a single precipitation line of identity exhibited by purified mono-specific polyclonal Vi-antibodies in the central well and wells containing highly purified Vi-CPS. However, the well containing the capsular polysaccharide of *Neisseria meningitidis* type A shows no cross-reaction with Vi-antiserum. In addition, anti-LPS antibodies of *Sal. typhi* Ty2 did not exhibit any precipitation line of reaction with Vi-CPS.

Determination of anti-Vi-CPS antisera titer

The antiserum titers of immunized rabbits were determined by passive hemagglutination method. From Table I, it can be inferred that antiserum titers for Vi-CPS were 1/32.

DISCUSSION

Being a homopolymer of (1-4) linked N-acetyl alpha D-galactosaminuronic acid acetylated at 0-3,[9] the immunogenicity of Vi-CPS is directly related to molecular size and configuration.[11,16] The downstream process of purification generally causes depolymerization of Vi-CPS to a non-immunogenic low molecular weight form.[20]

The present investigation was aimed to produce Vi-CPS in semi-industrial scale while retaining its molecular configuration, as native as possible, to be used in mass vaccination programs against typhoid fever in Iran.

As illustrated in Fig. 3 and Table I, the yield of the production was increased from 1.8 mg/L to 4.9 mg/L (total increase by 3.1 mg/L), when *Sal. typhi* Ty6s was adapted in submerged growth cultural conditions in modified Frantz fermentation broth, whereas the production rate of Vi-CPS reported by Elaine et al.[5] was 1.6 mg/L The extraction was done from live cells, and the product was highly purified, having more than 4.7 mMol O-acetyl, less than 10 mg protein and less than 20 mg nucleic acid as contaminants.

The elution profile of Vi-CPS in HPLC illustrates only one peak of high molecular weight (Fig.4) with a retention time of 11 minutes, which proves the purity of the product. Moreover, the Vi-CPS was eluted in high molecular weight aggregated in Sepharose 4B-CL. At least more than 50% of the recovered Vi-CPS was eluted before a KD value of 0.25.

There was no decrease in weight of guinea pigs and mice when the abnormal toxicity test was performed. Moreover, pyrogenic response tests of the rabbits were done and no rise in body temperature observed when 10, 20, 50 and 100 μg of purified Vi-CPS was injected intravenously.

Concerning the observation of the present study, Vi-CPS can be produced in semi-industrial scale while retaining its native configuration and immunogenic status. Therefore, Vi-CPS can be regarded as a safe, nontoxic and reliable immunogen to control typhoid fever in man.

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