

Simple Purification of Shiga Toxin B Chain from Recombinant *Escherichia coli*

OH, YOUNG PHIL, SEONG TAE JEONG, DAE-WEON KIM, EL-CHAE KIM¹,
AND KI-HONG YOON*

School of Food Science and Biotechnology, Woosong University, Daejeon 300-718, Korea

¹VaccTech Inc., P.O. Box 115, Bioventure Center in KRIBB, Yusong, Daejeon 305-600, Korea

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Abstract A plasmid expression vector of pEStx1 encoding a mature form of the B chain of the Shiga toxin was constructed without a signal peptide under the control of an inducible T7 promoter. The encoded protein was purified to 90% by simple heat treatment, and then further purified to 95% by Phenyl-Sepharose and DEAE-Sepharose chromatographies, all in a single day. Accordingly, this expression system and heat treatment could facilitate the rapid purification of gram-scale amounts of the Shiga toxin B subunit from recombinant *Escherichia coli* cells.

Key words: Shiga toxin, purification, heat treatment, overexpression

The Shiga toxin (Stx) of *Shigella dysenteriae* type 1 and homologous Shiga-like toxins (SLTs) of *Escherichia coli* O157:H7 can cause serious clinical complications in humans infected by these organisms [12]. Stx consists of A and B subunits (StxA and StxB, respectively). One StxA and five StxB polypeptide chains associate noncovalently with an apparent subunit stoichiometry. The crystal structure of Stx, the prototype toxin of the family, reveals a globular StxA subunit associated noncovalently with a crown-shaped pentamer of StxB subunits [6]. The StxA chain of the toxins inhibits eukaryotic protein synthesis by acting as an N-glycosidase, cleaving an adenine residue at nucleotide position 4,323 in the 28S rRNA of the 60S ribosomal subunit. A key functional role of the StxB subunit is to mediate the binding of the toxin to surface receptors on susceptible cells [10]. Although the StxB subunit alone has no *in vivo* cytotoxicity, it can bind to HeLa cells and prevent the cytotoxicity of a holotoxin [5]. Therefore, the StxB subunit is regarded as a candidate for the development of protein therapy or vaccine.

In the current work, an *E. coli* strain that hyper-produces StxB was constructed, and a rapid and simple large-scale purification scheme was developed.

High Expression of StxB in *E. coli*

A recombinant plasmid containing the StxB gene, designated as pEStx1, was constructed without a signal peptide coding sequence for high-expression of the gene in *E. coli*. Plasmid pSBC54 containing the StxB gene [2] was used as the template DNA for the polymerase chain reaction (PCR). Since the StxB gene has no suitable restriction sites that could be introduced into the expression vector pET24d(+) carrying a kanamycin-resistant gene as a selective marker, the StxB gene was amplified by PCR using primers with *Nco*I and *Eco*RI restriction sites. The sequences of the primers were 5-GCAAGTGCCATGGCGACGCCTGAT-TGTGTA-3 (upper primer for pEStx1), 5-CGCGAATTC-TCAACGAAAAATAACTTCGCT-3 (lower primer for pEStx1). The amplified fragment for the StxB gene consisting of 270 nucleotides was subcloned into pET24d(+) using *Nco*I and *Eco*RI, respectively. The plasmid pEStx1 (Fig. 1) was then transformed into *E. coli* BL21(DE3).

To investigate the effect of the growth temperature on StxB productivity and solubility in *E. coli* BL21(DE3)/pEStx1, the recombinant cells were grown and induced with 0.5 mM IPTG [4, 7, 9, 15, 16] at various temperatures [8, 11]. The expressed StxB protein was determined based on the difference between the total cell extracts and cell-free extracts on SDS-PAGE (data not shown). The StxB solubility was found to be dependent on the growth temperature from 20°C to 37°C, with the highest solubility at 20–25°C, whereas the productivity did not change significantly at different growth temperatures. At above 30°C, the expressed StxB existed mainly in an insoluble form.

Purification of Shiga Toxin B Subunit

The recombinant *E. coli* was grown at 20°C in an LB medium (2 liters) supplemented with kanamycin (50 µg/

*Corresponding author

Phone: 82-42-630-9742; Fax: 82-42-636-2676;
E-mail: ykh@lion.woosong.ac.kr

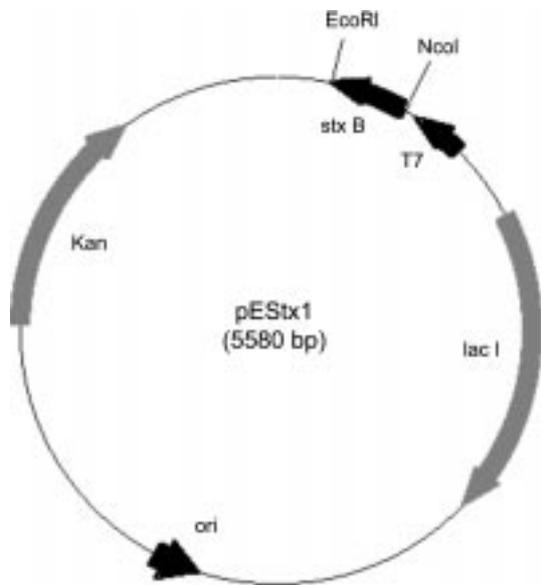


Fig. 1. Structure of expression plasmid pEStx1 containing the StxB gene (*stxB*). The restriction enzyme sites for introducing the StxB gene into pET24d(+) are indicated only. The arrows indicate the direction of transcription.

ml) until OD_{600} reached 0.5. After adding IPTG, the cells were allowed to grow for another 6 h, and then harvested by centrifugation. The cells were suspended in 200 ml of 50 mM sodium phosphate buffer (pH 8.0), disrupted by ultrasonic treatment, and centrifuged at $12,000 \times g$ for 1 h. The cell-free extract was then subjected to heat treatment for 30 min at different temperatures, ranging from 55 to $70^\circ C$ to test thermostability of the StxB protein. After centrifugation ($12,000 \times g$ for 20 min), the samples were analyzed by SDS-PAGE. As shown in Fig. 2, heat

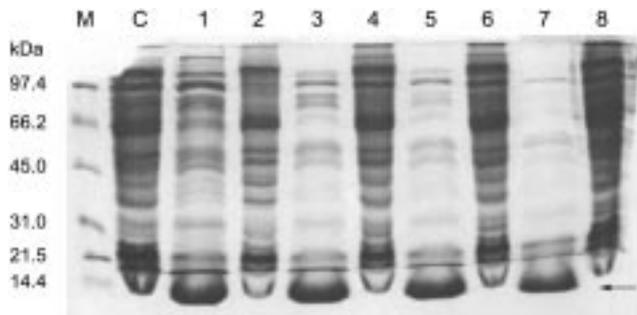


Fig. 2. SDS-PAGE of cell-free extracts after heat treatment. The cell-free extracts were treated for 30 min at 55, 60, 65, and $70^\circ C$, respectively, electrophoresed on 15% SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. Lanes: M, molecular weight markers; C, cell-free extract before heat treatment; 1, supernatant after heat treatment at $55^\circ C$; 2, precipitate after heat treatment at $55^\circ C$; 3, supernatant after heat treatment at $60^\circ C$; 4, precipitate after heat treatment at $60^\circ C$; 5, supernatant after heat treatment at $65^\circ C$; 6, precipitate after heat treatment at $65^\circ C$; 7, supernatant after heat treatment at $70^\circ C$; 8, precipitate after heat treatment at $70^\circ C$. Arrow indicates the StxB protein.

treatment at above $65^\circ C$ for 30 min removed almost all the *E. coli* proteins, except for the StxB protein, from the cell-free extract; thus approximately 90% purity of the StxB protein was obtained by the heat treatment. It is to be emphasized that this step was highly effective in purifying the protein, thereby greatly reducing the time and cost of purification.

To remove the remaining contaminants, the heat-treated supernatant was precipitated with ammonium sulfate to a final concentration of 30%, followed by loading the resulting supernatant on a Phenyl-Sepharose column (Pharmacia, Uppsala, Sweden) preequilibrated with 50 mM sodium phosphate buffer (pH 8.0) containing 30% ammonium sulfate. Thereafter, the ammonium sulfate concentration was decreased from 30% to 0%, and the bound StxB was eluted at the end of the gradient. The fractions containing the StxB protein were pooled and directly applied to the DEAE-sepharose column (Pharmacia, Uppsala, Sweden) which had been equilibrated with 100 mM sodium phosphate buffer (pH 8.0). Since the StxB did not bind to the column while the other contaminants were bound, the StxB protein was eluted during the sample loading. The unbound StxB protein was concentrated using a Microcon-3 (Amicon, Beverly, MA, U.S.A.) and stored at $-70^\circ C$. The final purification yield of the StxB was 30% (15 mg/l) from the cell-free extract (50 mg/l), estimated by the densitometry of polyacrylamide gels (Fig. 3).

Earlier methods to isolate the StxB subunit from the native Stx holotoxin involved multisteps or strong denaturants to separate the subunits [5, 17]. Such methods are time-consuming and labor-intensive, because renaturation

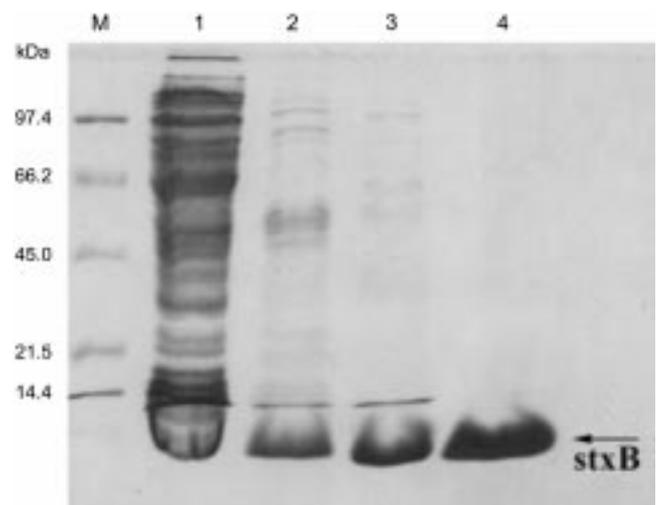


Fig. 3. SDS-PAGE of StxB protein during purification. The fractions at each stage of the StxB purification were electrophoresed. Lanes: M, molecular weight markers; 1, cell-free extract of *E. coli* BL21(DE3)/pEStx1; 2, supernatant after heat treatment for 30 min at a $65^\circ C$; 3, StxB protein after Phenyl-Sepharose chromatography; 4, purified StxB protein in DEAE-Sepharose unbound fractions.

procedures are required to purify the denatured StxB protein. Recently, several methods have been described for purifying StxB in recombinant *E. coli* [1, 3, 13, 14], and they include receptor analog affinity chromatography [3], sequential steps of DEAE-Sephacel chromatography, chromatofocusing, and Sephadex-G50 chromatography [13, 14], or sequential steps of ammonium sulfate precipitation, isoelectric focusing, and nondenaturing PAGE [1]. Accordingly, when compared with these methods, the heat treatment method described in the current study is cost effective and easy to scale up. In addition, the new method does not involve dialysis or a concentration step during the purification, thus making it possible to purify a gram-scale amount of StxB within one day. Therefore, the simplicity of the described method will increase the usefulness of the protein as a vaccine, either by itself or coupled to an appropriate polysaccharide antigen and therapeutic agent. In addition, due to its binding specificity, StxB also has the potential to be a carrier of other molecules in addition to the toxic StxA subunit.

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