Isolation of an *Escherichia coli* Heat Stable Enterotoxin (STb)-Alkaline Phosphatase Fusion Protein by Preparative Isoelectric Focusing

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Abstract

Preparative isoelectric focusing (IEF) with the Rotofor® cell was used to purify an *E. coli* heat stable enterotoxin (STb)-alkaline phosphatase (PhoA) fusion protein (STb-PhoA). The hybrid protein was genetically constructed to serve as a source of STb immunogen that could be readily purified. Following induction and expression of the T7-promoted gene fusion, bacterial cells were disrupted to release the hybrid protein product. The cell lysate was subjected to sequential ultra centrifugation, dialysis, and preparative isoelectric focusing. SDS-PAGE analysis of Rotofor separated cell material revealed that PhoA-containing fractions were substantially free of protein contaminants.

A single 4 to 5 hour Rotofor run in the pH 3–10 range yielded milligram quantities of highly purified protein. STb-PhoA-containing fractions were separated by size exclusion chromatography on Bio-Gel® P-100 gel, resulting in an essentially homogeneous preparation. The highly purified STb-PhoA protein was used to immunize rabbits and mice for polyclonal and monoclonal antibody production.

Methods

PREPARATION OF BACTERIAL LYSATE

The STb-PhoA fusion plasmid, pST-PhoA, is shown in Figure 1. Construction of the gene fusion is described in detail elsewhere.[1] Briefly, the weak endogenous STb promoter[2] was removed and replaced with a bacteriophage T7 promoter. The T7 promoted STb intermediate plasmid was digested with Bg1II and PstI to remove the carboxyl terminus of the STb gene. The codons removed by the restriction enzyme digestion were then replaced with synthetic oligonucleotides which included a 3′ in-frame overhang for a PhoA gene cassette obtained by PstI digestion of the bla-PhoA gene fusion containing plasmid pCH2.[3] After ligation, the fusion construct was used to transform *E. coli* HB 101.

Transformants were analyzed for plasmid DNA containing the appropriate restriction pattern. The newly constructed pST-phoA plasmid was then transformed into an *E. coli* HB 101 strain already harboring a plasmid (pGP1-2) encoding a thermally inducible T7 RNA polymerase.[4]

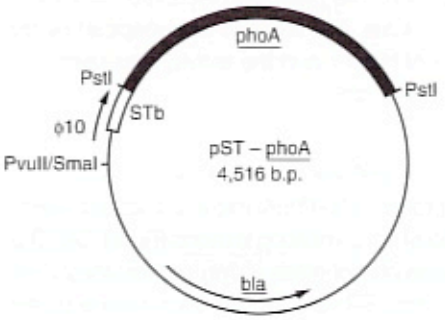

Fig. 1. Schematic representation of pST-PhoA drawn to scale. Phi 10 designates the bacteriophage T7 promoter. The direction of T7 directed transcription is denoted by the arrow.

Two liters of L medium[5] containing carbenicillin (100 µg/ml) and kanamycin (50 µg/ml) were inoculated with a 1:50 dilution of an overnight culture of *E. coli* HB101 (pGP1-2, pSTb-phoA). The culture was grown to mid-log phase (O.D. 1.0, 600 nm) at 30 °C, then shifted to 42 °C for 20 minutes to induce the pGP1-2 encoded T7 RNA polymerase. After 20 minutes at 42 °C, rifampicin (100 µg/ml) was added to inhibit *E. coli* RNA polymerase mediated transcription. After 15 minutes the culture was returned to 37 °C for 2 hours, during which time the T7 promoted STb-phoA was selectively expressed. The bacterial cells were harvested by centrifugation, washed in ice-cold Tris buffer (25 mM, pH 8.0), resuspended in 25 ml of the same buffer, and mechanically disrupted by passage through a French pressure cell. The broken cell material was centrifuged at 200,000 x g to pellet the particulate fraction, and the supernatant portion was prepared for IEF by overnight dialysis at 4 °C against water.

PREPARATIVE IEF OF THE STB-PHOA CONTAINING BACTERIAL LYSATE AND ANALYSIS OF THE FRACTIONATED PRODUCT

The dialyzed bacterial lysate (approximately 30 ml containing 288 mg of soluble bacterial protein) was mixed with Bio-Lyte® ampholytes (pH range 3–10; 40% w/v) at a final concentration of 1.5% in a total volume of 50 ml and stored on ice while the Rotofor unit was pre-electrophoresed for 30 minutes at 12 W constant power with ddH_2O to clean the components of the focusing chamber.

RUNNING CONDITIONS

The sample was loaded in the Rotofor cell as described in the users manual and run at 12 W constant power, 4 °C. Initial voltages were in the range of 450 V. Equilibrium was reached in 4–5 hours, at which time the voltage was approximately 900 V.

SAMPLE ANALYSIS

Twenty fractions were collected and their pH determined. Aliquots from each fraction were analyzed by SDS-PAGE and assayed for alkaline phosphatase activity in 96-well microtiter plates by mixing 20 µl of each Rotofor fraction, 40 µl of 5x diethanolamine buffer (catalog number 172-1063), and 40 µl of H_2O, followed, after mixing, by 100 µl of substrate solution (p-nitrophenyl-phosphate, 2 mg/ml in 1x diethanolamine buffer). The plate was incubated at room temperature for 15 minutes. The reaction was stopped by the addition of 25 µl of 2 N NaOH, and the activity was read in an ELISA plate reader at 405 nm.

Results

A single Rotofor run was sufficient to resolve the STb-alkaline phosphatase fusion protein (STb-PhoA) into 4–5 fractions which were substantially free of contaminating proteins (Figure 2A). The pH gradient and alkaline phosphatase activity profiles are shown in of Figure 2B. The hybrid STb and alkaline phosphatase protein have properties which allow separation by IEF. Wild-type *E. coli* alkaline phosphatase has a significantly more acidic pI (4.5) than the apparent pI of the fusion protein. The preparation shown in Figure 1 was generated from a PhoA proficient *E. coli* host. Hyper-expression of STb-PhoA achieved through the T7 expression system is readily detected in the assay used. The relatively low level expression of wild-type PhoA under these growth conditions is not detected. In preparations from strains lacking the hyper-expressed STb-PhoA fusion protein, the wild type PhoA is observed, but its position, relative to STb-PhoA, is 3–4 fractions toward the anode. Thus, the addition of 8 STb-derived basic residues[5] to alkaline phosphatase had the effect of moving the bulk of the STb-PhoA hybrid protein into fractions with an average pI of approximately 6.4. The observed shift in the pI of alkaline phosphatase allowed single step recovery of highly purified fusion protein.

The immediate goal of these studies was to produce a source of STb immunogen that could be readily assayed and purified; STb itself has neither of these properties. Genetic construction studies have been very successful in providing the means to selectively hyper-express the protein in question, but as shown in Figure 2, the starting material for the separation experiment was very complex. This type of purification problem is well suited for IEF, provided an appropriate gradient range is found.

A single 4–5 hour Rotofor run in the pH 3–10 range yielded milligram quantities of highly purified protein. The starting material for the run shown in Figure 2 contained 288 mg of total protein. Runs performed with slightly higher protein loads revealed no noticeable decrease in resolution. When an attempt was made to further purify the sample by refractionation in a

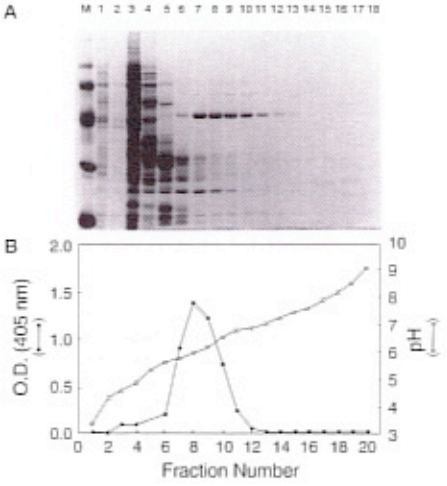

Fig. 2A. Analysis of bacterial lysate. SDS-PAGE analysis of Rotofor fractions 1–18. B. pH profile and relative alkaline phosphatase activity present in each Rotofor separated fraction.

narrow pH gradient (pH 5–8), the material was simply diluted without significantly increasing the level of purity. Another modification of the focusing conditions was made to initially run a narrow pH gradient in the pH 5–8 range, pool the fractions containing the fusion protein, add pH 3–10 range Bio-Lyte ampholytes, and rerun in the wider pH range. The first run gave very good separation of the cell extract, but distributed the fusion protein over 10 to 12 tubes. The second run in the wider pH range concentrated the fusion protein in fewer fractions. The original procedure and the modification gave comparable results, so the former, which required only the single run and thus less ampholyte, was adopted.

Conclusion

Preparative IEF using the Rotofor system was an effective method for the rapid isolation and near purification of an *E. coli* heat-stable enterotoxin (STb)-alkaline phosphatase fusion protein. A single Rotofor run combined with a single gel filtration step yielded a preparation of STb-PhoA which was suitable for use as an immunogen. Preliminary studies by western blot analysis indicate that polyclonal sera from rabbits hyper-immunized with the Rotofor purified STb-PhoA recombinant protein recognize native STb enterotoxin. Currently we are screening hybridomas generated from mice immunized with Rotofor-purified STb-PhoA for monoclonal antibodies to the wild-type protein toxin.

References

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