APPENDIX I

Purification of Endonuclease III from *Escherichia coli*

INTRODUCTION

The procedures here are modified from those published previously (*1, 2*).

MATERIALS AND METHODS

Materials

JM101 *E. coli* stock was obtained from New England Biolabs and maintained as a 50% glycerol stock at -80°C thereafter. pNTH10 EndoIII expression vector was obtained from Prof. O'Connor at City of Hope (Duarte, CA) (*2*). All buffers and culture media were prepared using standard procedures and sterilized by autoclave or sterile filter techniques (*3*).

Transformation

JM101 was freshly transformed with pNTH10 prior to each protein preparation. JM101 cells were streaked from a freezer stock to Luria-Bertani (LB) agar and incubated at 37°C overnight. A 1 mL LB culture was started from a single colony and grown up overnight at 37°C. 50 μL of the starter culture was used to inoculate a 10 mL LB culture. This culture was grown to the appropriate density at 37°C and made competent according to standard procedures (*3*). pNTH10 was transformed by electroporation at 1.7 kV or heat shock at 42°C. Transformed cells were allowed to recover at 37°C for 0.75-2 hours. Transformants were selected by plating on LB+ampicillan (amp) (50 μg/mL) followed by overnight incubation at 37°C.

Expression Test

From a single colony of JM101/pNTH10, a 1 mL LB+amp (50 μg/mL) culture was grown overnight at 37°C. 50 μL of the starter culture was used to inoculate a 50 mL LB+amp (50 μg/mL) culture. This culture was grown to an OD_{600} ~ 0.6-0.8. Isopropyl-β-*D*-thiogalactopyranoside (IPTG) was added to induce protein expression (125 μL, 0.2 M IPTG stock). 1 mL aliquots were removed at 2, 4, 5, and 6 hours postinduction. Cells were pelleted from these aliquoted and the pellet was resuspended in water and 2X SDS-PAGE buffer (0.09M Tris-Cl, pH 6.8, 20% glycerol, 2% SDS, 0.05% bromophenol blue, 0.1 M DTT). These samples were loaded onto a 4-15% Tris-HCl gradient gel and electrophoresed for 35 minutes at 200V. Gel was stained for 1 hour with BioSafe Comassie (BioRad) stain, destained overnight in water, and imaged.

Large-Scale Expression

A 1 mL LB+amp (50 μg/mL) culture was started from a single JM101/pNTH10 colony and grown up overnight at 37°C. This culture was used to start a 500 mL LB+amp (50 μg/mL) which was grown up overnight at 37°C. 50 mLs of this culture was used to start a 1L LB+amp (50 μg/mL) culture. This 1L culture was grown shaking at 220 rpm at 37°C until it reached an OD_{600} of 0.6-0.8. IPTG was added to each liter (0.5 mLs, 1M IPTG stock) and the cultures were grown an additional 4-6 hours at 37°C. 1 mL aliquots were removed before induction and at the end of the 4-6 hour induction period. These were analyzed as described above for successful induction of protein expression. Cells were transferred to centrifuge bottles and centrifuged at 3000 rpm for 20 minutes at 4°C. Supernatant is discarded and the pellet is resuspended in wash buffer (10 mM Tris, pH 7-8, 100 mM NaCl, 1 mM EDTA) and centrifuged again. The

pellet may be stored temporarily at -20°C. A typical yield is ~5g cell pellet/L culture. A typical protein prep is usually 8-16 L.

Cell Lysis

Cells are thawed on ice and resuspended in lysis buffer (250 mM KCl, 50 mM Tris, pH 8.0, 0.5 mM EDTA, 5% glycerol, 10 mM β-mercaptoethanol) at 4X the wet weight of the cell pellet (i.e., 25g cells in 100 mL lysis buffer). Note that in later preparations, the β-mercaptoethanol was omitted from all buffers without any adverse effects. Lysozyme was added at a final concentration of 1 mg/mL and phenylmethylsulfonyl fluoride (PMSF) was added at a final concentration of 0.14 mM (stock is 50 mM in isopropanol prepared just prior to use). This mixture is incubated on ice for 15 minutes. DNaseI and RNaseA are added at final concentrations of 5 μg/mL and 10 μg/mL, respectively. Solutions are incubated a further 30 minutes at room temperature and then centrifuged at 4°C for 20 minutes at 15,000 rpm in an SS-34 rotor. The supernatant is retained and stored on ice. From this point on, all manipulations are performed at 4°C or on ice unless otherwise specified.

Anion Exchange Chromatography

Quanternary methylammonium (QMA) resin (Sigma) was used to remove excess nucleic acids from the cell lysate. A QMA column was equilibrated with QMA buffer (250 mM NaCl, 10 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 5 mM β-mercaptoethanol, and 5% glycerol). Lysate was loaded onto the column and all fractions containing protein were collected.

Dialysis

QMA fractions are pooled and loaded into 10,000 MWCO dialysis tubing (Pierce) and dialyzed in dialysis tubing against the cation exchange column loading buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 0.1 mM EDTA, 5% glycerol) overnight.

Cation Exchange Chromatography

Sulfopropyl sepharose (SP) resin (Sigma) was used to perform the main purification step. The column volume used was 10% of the dialysate volume (i.e., if 300 mLs dialysate must be loaded, then a 30 mL column should be prepared). The SP resin was equilibrated with loading buffer and the dialysate was loaded onto the column. The loaded column was then rinsed with 5-10 column volumes loading buffer to remove any nonspecifically bound proteins. A gradient was run from 150 mM NaCl to 800 mM NaCl. All yellow bands were collected in 1-2 mL fractions.

Ammonium Sulfate Precipitation

SP column fractions were pooled in a beaker on ice over a stir plate. Ammonium sulfate was added to a final concentration of 1 g/mL (saturated) slowly over 20 minutes. The solution was stirred an additional 20 minutes on ice and then centrifuged at 15,000 rpm at 4°C for 30 minutes in an SS-34 rotor. Resulting pellet should be brown and the supernatant should be clear. Pellet was retained and resuspended in 1-3 mLs gel filtration loading buffer (1M NaCl, 10 mM Tris, pH 7.5, 0.5 mM EDTA, 10 mM β-mercaptoethanol).

Size Exclusion Chromatography

AcA54 resin (Sigma) was equilibrated with gel filtration loading buffer. The resuspended pellet from the previous step was loaded onto the AcA54 column and eluted with the loading buffer. All dark yellow fractions were retained. Purity was evaluated by SDS-PAGE (Figure A1.1).

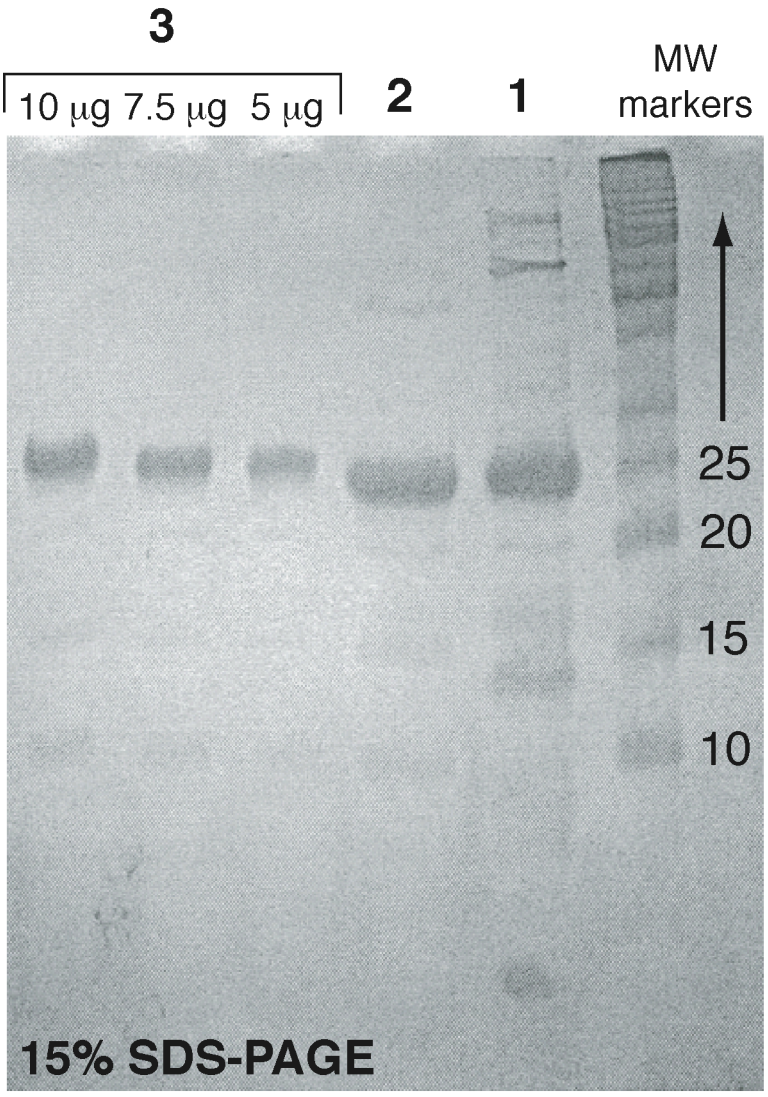
Concentration and Storage

Protein solutions were concentrated by either reverse dialysis with polyethylene glycol (PEG) in gel filtration loading buffer or by Centriprep 10 (Amicon) devices. Concentrated solutions are dialyzed into storage buffer (20 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1 mM EDTA, 20% glycerol) and stored in working aliquots at -80°C.

Summary

Figure A1.1 shows a representative SDS-PAGE gel after completion of the purification steps outlined here.

Figure A1.1. Purification of *E. coli* Endonuclease III.



1. After Phosphoultrogel cation exchange column

2. After AcA54 size exclusion column

3. After concentration and dialysis (MWCO ~ 10,000)

Yield = 2 mLs of ~ 200 μM

12 L synthesis

REFERENCES

1. O'Connor, T.R. (1993) *Nucleic Acids Res*. *21*, 5561.

2. Boiteux, S., O'Connor, T. R., and Laval, J. (1987) *EMBO J. 6*, 3177.

3. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.