Preparative Native PAGE Purification of Monomeric DAB_{389}-IL-2 Fusion Proteins from Bacterial Lysate

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Introduction

DAB_{389}-IL-2 is a genetically constructed fusion protein composed of the first 389 amino acid residues of diphtheria toxin (DT) fused to the polypeptide hormone, interleukin-2 (IL-2).[1,2] The first 389 amino acids of the DT contain all of the catalytic (C) and translocation (T) domains of the native toxin.[3] The IL-2 portion of the molecule selectively targets the fusion toxin to cells expressing the high affinity form of the IL-2 receptor.[2,4,5] Fragment A of DT is the enzymatic portion which, once delivered to the cell cytosol, catalyzes the adenosine diphosphate (ADP)-ribosylation of elongation factor 2. The fragment DT B translocation domain of the fusion toxin facilitates the delivery of fragment A to the cytosol. The goal of our research is to further understand the precise role played by the translocation domain in the delivery of fragment A to the cytosol. To this end, deletion and cassette exchange mutagenesis were performed on this fraction of the fusion toxin gene. Because of the tendency of some mutants to form dimers and higher order aggregates, it was necessary to devise a scheme for purification of native, monomeric forms of DAB389-IL-2 and the newly designed mutants. We report here the use of the Model 491 Prep Cell for the purification of active DAB_{389}-IL-2 monomer by continuous elution electrophoresis, from a non-denaturing polyacrylamide gel.

Methods

SAMPLE PREPARATION

The gene encoding DAB_{389}-IL-2 was re-cloned into the expression vector pETIId and the resulting plasmid was transformed into *E. coli* HMS174. Expression of the fusion toxin was under control of the T7 polymerase promoter and was induced upon addition of the coliphage derivative, CE6, which encodes T7 polymerase (Novagen). A 250 ml volume of cells, at $O.D._{600}$ = 0.8, was induced for 3 hours and then the bacteria were pelleted by centrifugation at 7,000 rpm for 10 min. The cell pellet was resuspended in 20 ml STET buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 8% glucose, 5% Triton®). Lysozyme was added to a final concentration of 12.5 µg/ml and the bacteria were incubated on ice for 1 h, at which time the sample was sonicated for 4 min, on ice. The sample was centrifuged at 11,500 rpm for 20 min at 4 °C. When expressed under these conditions the fusion toxin is insoluble and will remain in the pellet. Following centrifugation, the pellet was resuspended in 10 ml STET buffer and the sonication and centrifugation steps were repeated 3 times. The final pellet was resuspended in 5.0 ml denaturing solution (7 M guanidine hydrochloride, 100 mM Tris-Cl, pH 8.0, 10 mM EDTA) and dithiothreitol was added to a final concentration of 6 mM. The sample was sonicated as before and the protein concentration was determined by the Bradford method. The sample was resuspended to approximately 10 µg/ml in refolding buffer (50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 5 mM reduced glutathione, 1 mM oxidized glutathione) and incubated overnight at 4 °C. The recombinant protein was then concentrated approximately 20-fold using a Membrex Benchmark concentrator.

PREPARATIVE NATIVE PAGE

The appropriate acrylamide concentration for separation of monomer from aggregate forms of the protein was predetermined on non-denaturing polyacrylamide slab gels. A 20 ml volume of 7% acrylamide (30:0.8 acrylamide:bis) separating gel was poured into the 37 mm diameter gel tube of the Model 491 Prep Cell. After polymerization, 15 ml of a 4% acrylamide stacking gel was polymerized on top of the separating gel. Tris-glycine buffer (25 mM Tris, 200 mM glycine) was used for the running and the elution buffer. Forty ml of sample, containing approximately 7 mg of total protein, was mixed with 4.4 ml 10x loading buffer (325 mM Tris-Cl, pH 6.8, 50% glycerol), loaded onto the stacking gel, and electrophoresed at 20 W constant power. Bromophenol blue was omitted from the loading buffer as it interfered with the elution profile. The elution buffer was pumped at a rate of approximately 200 µl/min. Protein elution was monitored by UV absorbance.

SAMPLE COLLECTION

Using the above conditions, glutathione elutes from the gel after 3–4 hours of electrophoresis. Once elution of glutathione was detected, 4 min fractions (0.8 ml) were collected. The elution of DAB_{389}-IL-2 began at the tail end of the glutathione

peak (Figure 1). The protein concentrations of the fractions were determined and selected fractions were electrophoresed on native 7% gels to determine those which contained monomeric DAB_{389}-IL-2 (Figure 2). SDS polyacrylamide gel electrophoresis was used to determine full length fusion toxin (not shown). The best fractions with respect to full-length monomer were pooled and tested for cytotoxic potency in an *in vitro* cytotoxicity assay system.

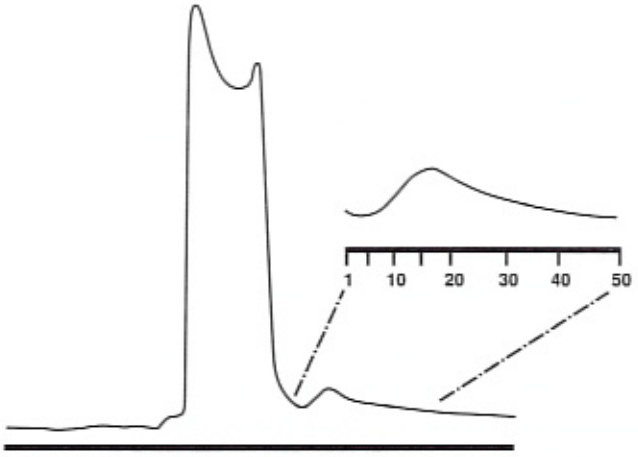

Fig. 1. DAB_{389}-IL-2 elution profile from the Model 491 Prep Cell using native polyacrylamide gel electrophoresis. The first peak contains the reduced and oxidized glutathione. The second peak contains the protein fractions eluted from the Model 491 Prep Cell. The indicated fractions were analyzed by native PAGE (inset).

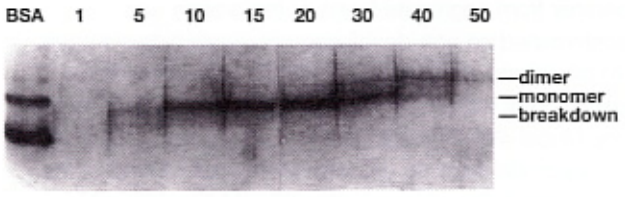

Fig. 2. Analysis of DAB_{389}-IL-2 fractions eluted from the Model 491 Prep Cell. A 50 µl volume of each indicated fraction was electrophoresed on a 7% native polyacrylamide gel. BSA was used as a control. Dimer, monomer, and breakdown products of the DAB_{389}-IL-2 are indicated.

Results and Discussion

Fractions 15–21 were pooled and tested for cytotoxicity. The level of cytotoxic potency recovered was consistent with values obtained for monomeric DAB_{389}-IL-2 that has been isolated using other methods. These results indicate that DAB_{389}-IL-2, isolated using the Model 491 Prep Cell, binds the IL-2 receptor on target eukaryotic cells, and is internalized. The enzymatically active fragment A is then delivered to the cytosol. For a typical experiment, 7 mg of protein is loaded onto the Model 491 Prep Cell and approximately 1.2 mg of monomeric DAB_{389}-IL-2 is collected in fractions 6–26 (Figure 2). Some of the loss may be due to protein that aggregates at the interface of the stacking gel. To avoid this, extra glycerol may be added to the sample. DAB_{389}-IL-2 monomer has been isolated by other methods such as HPLC and ion exchange chromatography. However, monomeric forms of some of the mutants could not be isolated with these methods. Following the above protocol, we are able to routinely isolate monomeric forms of DAB_{389}-IL-2 mutants that we were unable to purify using other methods.

References

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