Purification and On-Column Refolding of Polyhistidine-tagged *Staphylococcus aureus* α-hemolysin Inclusion Bodies on the Profinia™ Protein Purification System

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Introduction

Bacteria are widely used as hosts for production of recombinant proteins that do not require post-translational modification, such as glycosylation, for bioactivity. With its well-defined genetics, highly efficient host-vector expression systems, and well-established protein purification methods, *E. coli* is usually the first choice for expression of recombinant proteins. However, high expression levels of proteins from strong promoters on high-copy-number plasmids can often result in the formation of insoluble protein aggregates known as inclusion bodies.

Although protein expression in the form of inclusion bodies is often considered undesirable, their formation can be advantageous. The main advantages associated with the formation of inclusion bodies are (1) expression at a very high level; expressed protein can represent more than 30% of the cellular proteins in some cases, (2) easy isolation of the inclusion bodies due to differences in size and density compared to some contaminants, (3) low degradation of the expressed protein due to the inclusion body's resistance to proteolysis, (4) high homogeneity of the protein of interest in the whole lysate before purification, which helps in reducing the number of purification steps, and (5) ability to overexpress toxic proteins especially for large-scale production.

The recovery of bioactive proteins from inclusion bodies involves four steps: isolation of inclusion bodies from *E. coli* cell extracts, solubilization of protein aggregates, refolding, and purification of the solubilized protein. Among these steps, refolding is the most crucial step and requires careful consideration for proper recovery of bioactive protein.

We used *Staphylococcus aureus* α-hemolysin (α-HA) as a model for expression and purification. This protein is a 293 amino acid toxin (Fussle et al. 1981) that is not easily purified as a recombinant protein. Secreted as a water-soluble monomer, the toxin binds the cytoplasmic membrane of eukaryotic target cells where it forms transmembrane pores of heptameric stoichiometry called "pre-pores" (Gouaux et al. 1994, Montoya and Gouaux 2003). Subsequently, the pre-pores transform into the final pores by membrane insertion of an amphipathic β-barrel, which comprises the "central loop" domains of all heptamer subunits, causing cell lysis (Figure 1). Although several protocols have been developed for expression and purification of the active wild type protein (Lind et al. 1987, Vandana et al. 1997, Cheley et al. 1997), all these methods involve multiple tedious and time-consuming steps.

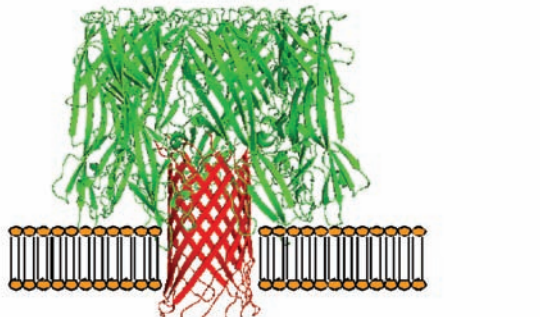

Fig. 1. Structure of α-HA. Soluble monomers of α-hemolysin bind to the membrane of eukaryotic cells to form heptameric pre-pores (green). Insertion of an amphipathic β-barrel (red) completes the pore formation.

In this tech note, we describe a protocol to purify and refold *Staphylococcus aureus* α-HA expressed as inclusion bodies in *E. coli* in a single automated step using the Profinia protein purification system. The inclusion bodies were denatured in urea, the solubilized protein was bound to an IMAC support, refolded while bound to the chromatographic support, and recovered as an active protein following a desalting step. This protocol is rapid and efficient for large-scale preparation of fully functional α-HA from inclusion bodies.

Methods

Overexpression of His-αHA

His-αHA was expressed in *E. coli* BL21 DE3 harbouring the pET28a His-αHA plasmid grown in 250 ml TB (12 g/L tryptone, 24 g/L yeast extract, 17 mM KH_2PO_4, 72 mM K_2HPO_4, glycerol 0.4 %) at 37°C, in the presence of 1 mM IPTG for 4 hr. IPTG induction was performed when A_{600} nm reached 1.2–1.4.

Recovery of Inclusion Bodies

Bacteria were harvested by centrifugation, resuspended in 200 ml Tris-HCl 50 mM, EDTA 1 mM, NaCl 100 mM, pH 8, and broken in an Avestin EmulsiFlex C-3 homogenizer (Avestin, Inc.). The pellet was recovered by centrifugation at 12,000 x g for 30 min at 4°C and membrane proteins were solubilized using 50 ml Tris-HCl 50 mM, EDTA 10 mM, NaCl 100 mM, Triton X-100 0.5 %, pH 8, for 18 hr at 4°C. The inclusion bodies were recovered by centrifugation at 30,000 x g for 30 min at 4°C and solubilized by incubation in 100 ml Tris-HCl 20 mM, pH 8, urea 8 M for 18 hr at 4°C. The soluble fraction was clarified by centrifugation at 30,000 x g for 30 min at 4°C.

Purification and Refolding of His-αHA

Purification and refolding of His-αHA was performed on the Profinia protein purification system (Bio-Rad Laboratories, Inc.) using the preprogrammed native IMAC + desalting method and the Profinia native IMAC buffer kit. Six ml of the soluble fraction, corresponding to 15 ml of bacterial culture, were added with 44 ml of Tris-HCl 20 mM, pH 8, urea 8 M, and then loaded on a 5 ml Bio-Scale™ Mini Profinity™ IMAC cartridge (Bio-Rad) at 2.5 ml/min. Refolding of the protein took place during the integrated washing step, with 6 column volumes of urea-free buffer. The protein of interest was eluted and desalted using the integrated desalting step on a 50 ml Bio-Scale Mini Bio-Gel® P-6 desalting cartridge (Bio-Rad).

Hemolytic Assays

The purified His-αHA protein was assayed at a final concentration of 20 µg/ml for hemolytic activity against washed defibrinated rabbit erythrocytes (rRBC) diluted 1/20 in PBS. After 30 min of incubation at 30°C, the rRBC were pelleted by centrifugation and hemolysis was assessed by measuring the hemoglobin released in the supernatant at 540 nm.

Results and Discussion

Conventional methods for refolding insoluble recombinant proteins from inclusion bodies often include time-consuming protocols using dialysis and/or dilution steps before or after the purification steps. Previously, we purified the His-tagged staphylococcal toxin under denaturing conditions followed by drop-wise dilution into a cold refolding buffer containing a detergent (Filée et al., unpublished data). The refolding was performed at a low protein concentration to prevent aggregation. After refolding was completed, the protein solution was dialyzed several times against PBS buffer in order to remove imidazole, urea, and the detergent before it was concentrated. The total time spent to perform this procedure was about 60 hr. Although this method is commonly used in small-scale refolding studies, it is both time- and buffer-consuming. In addition, this technique is accompanied by loss of protein during filtration and concentration of large volumes.

In this study, we describe a rapid method to obtain purified bioactive His-tagged staphylococcal toxin in a single step. Polyhistidine tagging of recombinant proteins makes it possible to perform on-column refolding and purification in a single step. Binding of the unfolded protein takes place because the presence of denaturant does not reduce the affinity of the polyhistidine tag for metal ions. The subsequent wash and elution steps use buffers that do not contain urea allowing on-column refolding of the protein. Refolding proteins immobilized on a support is efficient because it minimizes intermolecular interactions that can lead to protein aggregation. Proper refolding of the protein can be confirmed using a functional assay.

Using the method described here, we purified 3.5 mg of His-αHA in less than one hour (Figure 2). The purified His-αHA (Figure 3, lane 3) was devoid of any precipitate, indicating that no aggregation occurred.

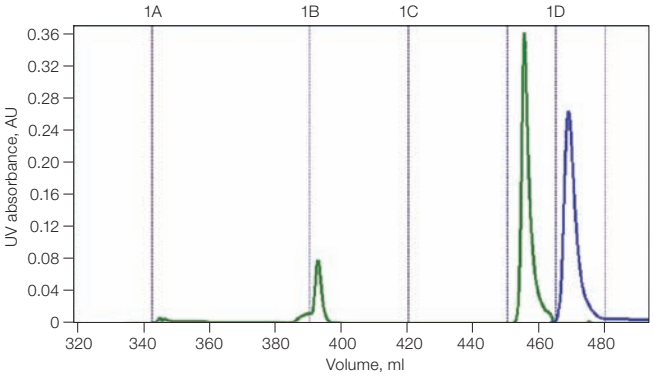

Fig. 2. Chromatograms showing the purification of refolded His-αHA using the native IMAC + desalting method on the Profinia system. The absorbance at 280 nm is monitored both at the output of the Profinity IMAC cartridge (green line) and the desalting cartridge (blue line).

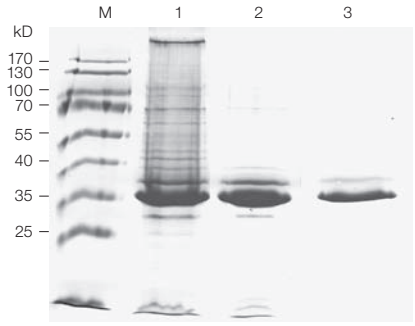

Fig. 3. SDS-PAGE showing production, purification of refolded His-αHA using the native IMAC + desalting method on the Profinia system. Lane M, molecular weight markers; lane 1, *E. coli* total extracts containing overexpressed insoluble His-αHA; lane 2, extracted and urea-solubilized inclusion bodies; lane 3, purified and refolded His-αHA. Only one third of the sample that is equivalent to lane 2 was loaded.

His-αHA purified and refolded on a Bio-Scale Mini Profinity IMAC cartridge using the Profinia protein purification system shows a hemolytic activity similar to the protein obtained using the dilution-based refolding protocol (Figure 4).

It is worth mentioning that the wash step could be performed as a two-step procedure: a first wash could be performed using a buffer containing urea (<6 M) before a second wash using a buffer containing no urea. This may provide more gentle conditions for the protein to refold. Because we obtained functional His-αHA using the simple purification procedure described here we did not try this additional step in our current protocol so the effect of a two-step procedure on the refolding of His-αHA is not known.

Alternatively, multiple wash steps with decreasing concentrations of urea can be done. In this case, manual replacement of the buffers after each wash step is needed. Washing the column in multiple steps should make the renaturation process more effective.

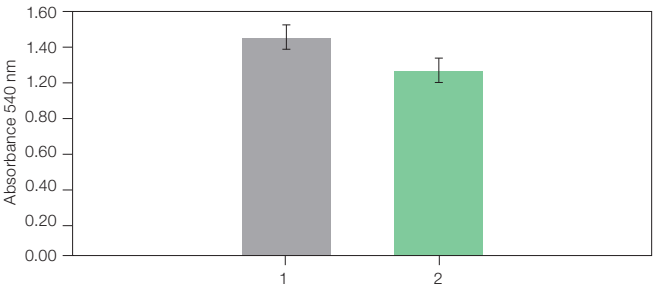

Fig. 4. Hemolytic activity of His-αHA. 1, hemolytic assay performed with the His-αHA prepared using a drop-wise dilution method. 2, hemolytic assay with refolded His-αHA purified with the Profinia protein purification system. The assays were performed by incubation of the toxin (20 µg/ml) with 1 ml of a 5% defibrinated rRBC suspension in PBS, pH 7.3, for 30 min at 37°C. The hemoglobin release was calculated from absorbance measurement at 540 nm.

Conclusions

In this tech note, we have described a protocol that permits both purification and refolding of a protein overexpressed in *E. coli* in a single automated step. This protocol uses IMAC to achieve simultaneous purification and refolding of recombinant His-αHA. The other valuable feature of our approach involves the fast and fully automated process of these two steps made possible by the Profinia purification system using the native IMAC + desalting method. Instead of spending 60 hr for the whole purification including tedious dilution, dialysis and concentration steps, the described procedure took only about 90 min for the whole process. Its efficiency demonstrates that the Profinia purification system is a powerful tool to tackle problems in downstream procedures and to generate refolded functional proteins.

References

Cheley S et al. (1997). Spontaneous oligomerization of a staphylococcal alpha-hemolysin conformationally constrained by removal of residues that form the transmembrane beta-barrel. Protein Eng 10, 1433-1443.

Fussle R et al. (1981). On the mechanism of membrane damage by *Staphylococcus aureus* alpha-toxin. J Cell Biol 91, 83-94.

Gouaux JE et al. (1994). Subunit stoichiometry of staphylococcal alpha-hemolysin in crystals and on membranes: A heptameric transmembrane pore. Proc Natl Acad Sci USA 91, 12828-12831.

Lind I et al. (1987). Purification of alpha-toxin from *Staphylococcus aureus* and application to cell permeabilization. Anal Biochem 164, 84-89.

Montoya M and Gouaux E (2003). Beta-barrel membrane protein folding and structure viewed through the lens of alpha-hemolysin. Biochim Biophys Acta 1609, 19-27.

Vandana S et al. (1997). The role of the amino terminus in the kinetics and assembly of alpha-hemolysin of *Staphylococcus aureus*. J Biol Chem 272, 24858-24863.

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