

Hemolytic Activity of Truncated Hemolysin A Variants

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ABSTRACT

Proteus species are second only to *Escherichia coli* as the most common causative agent of urinary tract infections and many harbor several virulence factors that provide inherent uropathogenicity. One of the virulence factors is a hemolysin system comprised of hemolysin A and hemolysin B. Hemolysin B secretes hemolysin A from the periplasmic space where it resides in an inactive state, through the outer membrane into the surrounding extracellular environment. Upon hemolysin B dependent secretion, hemolysin A functions as a hemolysin and disrupts neighboring host cell membranes. In order to describe the mechanism by which hemolysin A is activated for pore formation, we have constructed, expressed, and purified a series of amino terminal truncates and cysteine mutants of hemolysin A capable of complementing the non-secreted full length hemolysin A and restoring hemolytic activity. All of the amino terminal truncates of hemolysin A were shown to activate hemolysis two to three-fold.

INTRODUCTION

Proteus species have been implicated in a number of nosocomial and opportunistic infections. They present most often as urinary tract infections in patients with urologic abnormalities or catheters, but even in a normal host *Proteus* species are second to *Escherichia coli* (*E. coli*) as the leading cause of urinary tract infections caused by Gram-negative rods (1, 2). Proposed virulence factors of *Proteus* include urease, pili, swarming motility, iron acquisition, IgA protease, and hemolysin. Collectively, these factors provide *Proteus* inherent uropathogenicity (7).

The hemolysin system within *Proteus* has been shown to be a member of the two-partner secretion (TPS) pathway, in which a large, exoprotein is secreted into the environmental milieu (Figure 1).

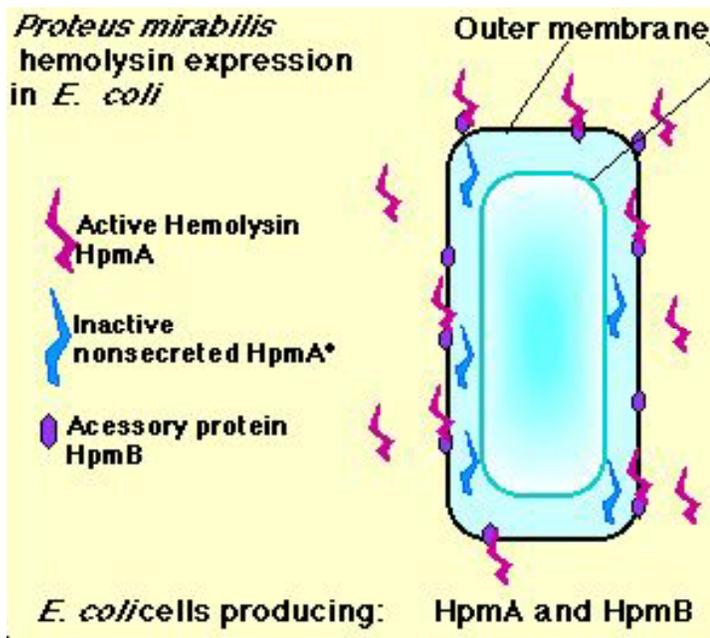


Figure 1. Hemolysin expression system within *Escherichia coli*.

All TPS pathways utilize an amino terminal module termed the “secretion domain” within the large exoprotein (termed the A-component) and a channel forming β -barrel transporter protein (termed the B-component) (5). HpmA and HpmB from *Proteus* are analogous to the A and B-components in the TPS system. The B-components act as specific receptors for the A-component secretion signals and activate their respective hemolysin/cytolysin concomitant with export through the outer membrane (6). Coupling secretion and activation ensures host cell survival by minimizing the formation of an internally active pore-forming A-component.

Within the amino terminal domain of HpmA, two cysteine residues arranged in a CxxC pattern were noticed. Cysteines arranged in this fashion have been shown to be involved in disulfide bond isomerization and metal binding (3, 4, 8). To determine the role of these two cysteines residues during the complementation of hemolytic activity, each was mutated into a serine residue. The resultant HpmA variants were termed C144S and C147S. Both of these variants, as well as two native HpmA variants (HpmA265 and HpmA358) were expressed, purified and analyzed for hemolytic activity.

MATERIALS & METHODS

Protein expression and purification

Plasmids harboring either *hpmA265*, *hpmA358*, *hpmA C144S* or *hpmA C147S* or *hpmB* were co-transformed into *E. coli* B834 (DE3) for over-expression experiments. The C144S and C1447 mutants were constructed from a template plasmid harboring *hpmA265*. Cell cultures experiments were conducted in 2.8 L Fernbach flasks harboring 1.4 L of Luria-Broth supplemented with 30 g/mL kanamycin and 30 g/mL chloramphenicol. Protein expression was induced at an OD⁶⁰⁰ of 0.8 for five hours via the addition of 1 mM with isopropyl-beta-D-thiogalactopyranoside. Cellular pellets were harvested via centrifugation at 20,000 x g for 15 minutes. Each HpmA variant was purified from the resulting supernatant using Ni²⁺-NTA chromatography. After loading the supernatant to a 25 mL Ni²⁺-NTA column and washing with Buffer A (50 mM sodium phosphate pH 7.8, 300 mM NaCl) to baseline at 280 nm, the contaminant proteins were removed via washing with Buffer B (50 mM sodium phosphate pH 7.8, 300 mM NaCl, 60 mM imidazole). Highly purified HpmA265, C144S, C147S, or HpmA358 were eluted with Buffer C (50 mM sodium phosphate pH 7.8, 300 mM NaCl, 400 mM imidazole) (Figure 2). The purified HpmA mutants were then concentrated to a final concentration of 10 mg/ml using a Savant SpeedVac and subsequently dialyzed into 10 mM Tris-HCl pH 7.5 for storage.

SDS-PAGE analysis

Each purified form of HpmA used for the hemolytic assays was analyzed under SDS-PAGE (Figure 2). Approximately 1 - 5 g of purified protein was loaded onto a 12% gel for SDS-PAGE analysis. The gel was run at 100 V for 20 minutes, followed by 200 V for 1 hour. The gel was stained with Coomassie Blue (0.2% coomassie blue R-250, 7.5 % acetic acid, 50 % ethanol) for one hour and destained (35% methanol, 15% acetic acid) overnight.

Hemolytic assays

The hemolytic complementation assay was performed by adding 1 μ g of purified HpmA (HpmA265, HpmA358, C144S, or C147S) into an assay tube containing HpmA (Sonicate), sheep red blood cells, and PBS. As negative controls, sonicated recombinant strains RAU 126 (full length HpmA - non secreted), HMS174 (DE3) (no *hpmA*), and purified HpmA variant alone were run side by side during the complementation assay. In addition, sheep red blood cells were monitored for spontaneous hemolysis (Figure 3).

RESULTS

The metal-chelate purification scheme provided a highly purified pool of HpmA variant (Figure 2).

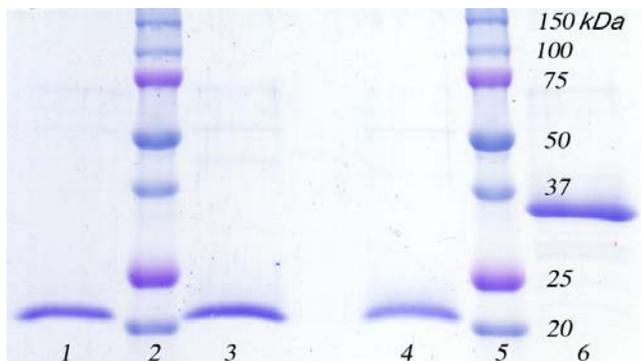


Figure 2. SDS-PAGE results for hemolysin purification schemes. Lane 1 - HpmA265, Lanes 2 and 5 - molecular weight markers, Lane 3 - C144S, Lane 4 - C147S, and Lane 6 - HpmA358.

The trend for total protein yield seems to indicate cysteines being favored at positions 144 and 147. The net yield for the HpmA265 form after cysteine replacement at either 144 or 147 drops from 31 mg to between 10 - 13 mg (Table 1). The purified HpmA variants (Figure 2) were utilized to complete a series of hemolytic complementation assays (Figure 3).

Table 1. Hemolysin A variant protein yields

| Sample | Total Protein (mg) |
|----------|--------------------|
| HpmA 265 | 31.88 |
| HpmA 358 | 15.75 |
| C144S | 10.67 |
| C147S | 13.67 |

Spontaneous hemolysis of sheep red blood cells alone was minimal throughout the course of study. In addition, sonicate prepared from HMS 174 (DE3) provided no measurable hemolytic activity. HMS 174 (DE3) was the *E. coli* strain used as the host for expression of full length non-secreted HpmA denoted as sonicate (Figure 3). The hemolytic complementation assay notes that inactive full length HpmA becomes activated in the presence of purified HpmA265, HpmA358, and even the cysteine mutants C144S and C147S (Figure 3).

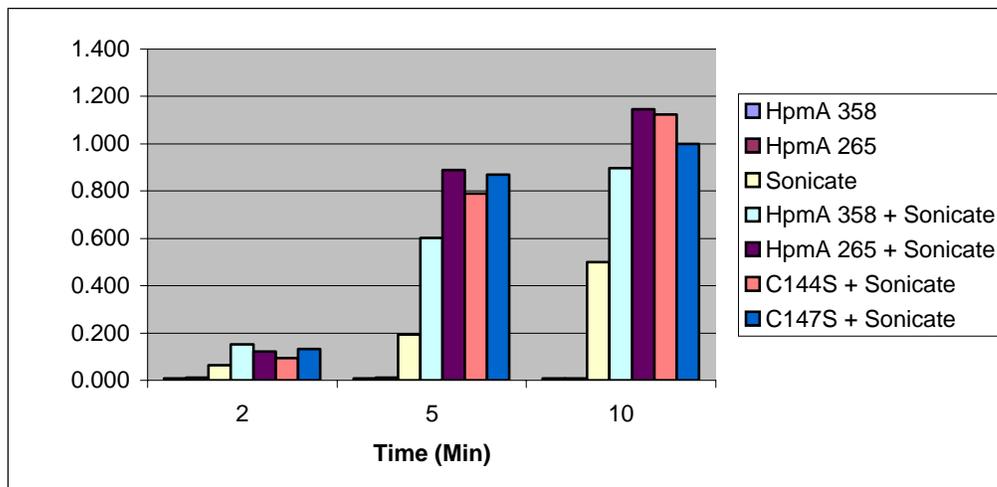


Figure 3. Hemolytic complementation assays

DISCUSSION

For the following discussion, the ten-minute time interval will be used (Figure 3). Neither HpmA265 nor HpmA358 alone were shown hemolytically competent (Figure 3 - far left blue and red bars). The loss of the carboxy terminal domain from these variants has eliminated the pore-forming region. However, based upon the fact that we were able to purify each of the four truncated forms of HpmA (HpmA265, HpmA358, C144S, and C147S) they all are HpmB secretion competent. Recall that in the presence of HpmB within the outer membrane of *E. coli*, HpmA will be secreted to the external environment (Figure 1). The data also suggest that non-HpmB secreted HpmA from the sonicate has a low level of hemolytic activity (Figure 3- yellow bar). If the two native HpmA truncates (HpmA265 and HpmA358) were mixed with the sonicate, a 2 to 3-fold increase in hemolytic activity was observed (Figure 3 - cyan and maroon bars).

Previous sequence analysis has noted a pair of cysteines arranged in a classic CxxC pattern. This arrangement of cysteine residues has been noted in enzymes involved in disulfide isomerization and metal binding (3,4, 8). Because these were the only two cysteines within the entire 1557 amino acid sequence we mutated these into serine residues, purified the resultant mutants and analyzed each for hemolytic activity. The hemolytic activity data indicates that neither cysteine replacement seems critical for the maintenance of hemolytic activity (Figure 3 - orange and royal blue bars). However, it could still be plausible that either C144S or C147S has less activity under other experimental conditions. For example, these two mutants may be more susceptible to denaturation at elevated temperature, pH or salt concentration and additional experiments will need to be conducted to test these parameters.

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