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Molecular Studies on C-terminus of *Helicobacter pylori* Ferritin

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Abstract

The current study was conducted with the main objective to minimize the length of the E-helix of Hpf in order to assemble into 24-mer for possible expression with heterologous proteins for the development of universal vaccine development. Hpf^{146}, Hpf^{148}, Hpf^{150}, $Hpf151^{N147}$, Hpf^{151}, Hpf^{152}, Hpf^{153} genes were amplified by PCR using the sense and antisense primers. All Hpf truncations were cloned into pET-Duet vector between BamHI and XhoI by creating an N-terminal His tag. The pET-Duet vector containing the wild-type Hpf gene was used for amplificat--ion of all truncations. The bacteria were grown at 37°C, and the plasmid was purified by using a Gene All plasmid mini kit (General Biosystems, Korea), which was analyzed by agarose gel electrophoresis. The clone obtained was confirmed by sequencing. In order to ensure the desired truncations and mutation, sequence was verified via automated DNA sequencing. Proteins were expressed in *E. coli* BL21/ DE3. Study findings delineated that all the three constructs *viz.* $Hpf\text{-}151^{N147}$, Hpf-151 and $Hpf\text{- }full^{L151A}$ showed part of the protein is assembled indicating that at least 151 residues are necessary for its assembly into a 24-mer. Leu151 and Leu153 are the hydrophobic residues of the E-helix, might control the acute angle between the D and E helices formed at the 4-fold axis. The $Hpf\text{-}full^{L151A}$ mutant did not disrupt the assembly, and the purified mutant protein behaved similar to native protein. Leu151 and Leu153 are the hydrophobic residues of the E-helix, might control the acute angle between the D and E helices formed at the 4-fold axis. The $Hpf\text{-}full^{L151A}$ mutant did not disrupt the assembly, and the purified mutant protein behaved similar to native protein.

Keywords: *Helicobacter pylori*, Ferritin, E-Helix, $Hpf\text{-}151^{N147}$, Hpf-151, $Hpf\text{- }full^{L151A}$

Introduction

Ferritin, a biomineralizing protein, has a highly conserved structure from bacteria to higher organisms. Ferritin plays a crucial role in maintaining the iron balance of organisms. Ferritin self-assembles into 24-mer spherical shells, molecular mass ranging from 450 to 500 kDa, which is arranged in an octahedral (432) symmetry, to form a nanocage architecture. The structure has six 4-fold axes, eight 3-fold axes, and twelve 2-fold axes. The 3-fold symmetry channels are involved in the transport of iron from outside to the inside of the protein in eukaryotes (ref), whereas the 4-fold symmetry channel serve as a pathway for iron translocation in *Helicobacter pylori* ferritin (Hpf) and prokaryotes (Cho et al. 2009). Each ferritin subunit (~19 kDa) consists of four-α-helix bundle containing two antiparallel helix pairs (A, B and C, D) and a fifth short helix (E helix). The E helix is located at one end of the bundle, rich in hydrophobic residues, making an angle at about 60° to the long axis of each subunit bundle, which is believed to be involved in stabilizing the 24-mer by hydrophobic interactions and maintaining the 4-fold symmetry axes (Lv et al. 2014).

Protein-based drug delivery systems are highly preferred over liposome based or direct administering of the drugs. The nontoxicity, stability, and the important biological functions of ferritin make it a very useful and versatile drug delivery system (Jutz et al. 2015). The ability of ferritin to self-assemble into nanoparticles makes ferritin well-suited for antigen presentation and immune stimulation. Ferritin has been the focus of much recent attention as part of drug delivery studies. Several proteins including viral proteins fused with ferritin is suitable for expression in prokaryotic cells, unless proteins are subjected to mammalian glycosylation and other post-translational modifications. Structural analysis of ferritins indicates that it is possible to insert a heterologous proteins at 3-fold and also at 4-fold with modification at the C-terminus through genetic engineering. Previously, hemagglutinin (HA) was fused to Hpf to present trimeric HA nanoparticle at the 3-fold axis (Kanekiyo et al. 2013). Fusion of proteins at the 4-fold axis is only possible after completely removing or truncating the E-helix. Expression of Hpf after removing the entire E-helix was found to prohibit nanoparticle assembly, which is in agreement with the previous studies where assembly disrupted due to alteration of residues involved in the contacts along the 4-fold symmetry axes. (Ingrassia et al. JBC). In the present study, an effort has been made to minimize the length of the E-helix of Hpf in order to assemble into 24-mer for possible expression with heterologous proteins for the development of vaccine.

Methods

Subcloning and mutagenesis

Hpf^{l46}, Hpf^{l48}, Hpf^{l50}, $Hpf151^{N147}$, Hpf^{l51}, Hpf^{l52}, Hpf^{l53} genes were amplified by PCR using the sense and antisense primers (Table.1). All Hpf truncations were cloned into pE T-Duet vector between BamHI and XhoI by creating an N-terminal His tag. The cloning strategy was such that the expressed protein included a hexa-histidine tag at the N-terminus. The clone obtained was confirmed by sequencing. The pET-Duet vector containing the wild-type Hpf gene was used for amplification of all truncations. The bacteria were grown at 37 °C, and the plasmid was purified by using a Gene All plasmid mini kit (Ge neral Biosystems, Korea), which was analyzed by agarose gel electrophoresis. In order to ensure the desired truncations and mutation, sequence was verified via automated DNA sequencing at Macrogen Co. (Seoul, Korea).

Expression and purification

Proteins were expressed in *E. coli* BL21/DE3 (Novagen, San Diego, CA, USA). Transformed bacteria were used to inoculate sequentially increasing volumes of Luria-Bertani (LB) growth media containing 100 mg/mL ampicillin at 37ºC. Upon reaching OD_{600}= 0.6, protein expression was induced by 200mM isopropyl 1-thio-β-galactopyranoside, which was followed by 12-16 h of incubation at 20ºC. Cells were harvested by centrifugation (9700 g) for 10 min. Cells were suspended with lysis buffer 50 mM Tris, pH 8.0, 200 mM NaCl, and 10 mg lysozyme. Cells were lysed by sonication (vibra cell, Sonics, Newtown, CT, USA) and debris were removed by 40 min centrifugation (20,000 g) at 4ºC, and the soluble fraction was loaded onto a preequilibrated metal chelate column (Ni-NTA (Qiagen, Valencia, CA, USA) in 20 mM Tris, pH 8.0 at a flow rate of 1 mL/min. The column was washed with 5 mM imidazole, until a stable base line was achieved to remove nonspecific proteins. After a step elution was performed by buffer A supplemented with 50 to 125 mM imidazole, the fractions containing the Hpf were pooled. Size exclusion chromatography (SEC) was then performed using a preequilibrated Superdex 200 analytical grade column (GE Healthcare, Piscataway, NJ, USA). Protein was eluted with 20 mM Tris, pH-8.0 and 100mM NaCl. Pooled fractions were concentrated and stored in -80ºC.

Results and Discussion

Hpf C-terminal truncations

We designed several truncations after helix-D to remove the entire E-helix, Hpf-146, Hpf-148, and Hpf-150 (Figure.2A). When these truncations were expressed, none of them showed the assembly into a 24-mer, whereas Hpf-151 with asparagine insertion at 147th position appeared by accident termed as Hpf-151^{N147}, showed small part of the protein assembled into 24 mer (Figure 1). Native PAGE showed a predominant band of Hpf-151^{N147}, clearly showing the assembly into a 24-mer. Apart from the 24 mer, smaller assemblies are also observed on native PAGE, those could be products of disassembly of the 24-mer.

Next, we made another construct, Hpf-151, which was also found to assemble small portion protein into 24 mer. Major portion of the protein from Hpf-151^{N147} and Hpf-151 are running higher than Hpf-full on native PAGE. Most of the protein might have aggregated or not stable like Hpf-full. These truncations showed heterologous species including large aggregates. Hpf-full is tightly packed and stable compared to Hpf truncations due to the E-helix, which provide hydrophobic lining of leucine residues. All the three constructs (Hpf-151^{N147}, Hpf-151 and Hpf-full^{L151A} showed part of the protein is assembled indicating that at least 151 residues are necessary for its assembly into a 24-mer. Leu151 and Leu153 are the hydrophobic residues of the E-helix, might control the acute angle between the D and E helices formed at the 4-fold axis. The Hpf-full^{L151A} mutant did not disrupt the assembly, and the purified mutant protein behaved similar to native protein. Mutational studies of hydrophobic residues at the entire E-helix and structural studies might provide further clues of its role in the assembly.

Sequence analysis and structural comparison among ferritins

Hpf shows significant sequence identity with those from eubacterial ferritins, *V. cholerae*, *E. coli*, *C. jejuni*, *C. tepidum*, *T. maritima*, *P. furiosus* (between 30-63%) (Arenas-Salinas et al. 2014) (Figure.2B). The *H.pylori* showed highest similarity with *Campylobacter jejuni* and sequence identity of about 63%, whereas *E.coli* is the least conserved having 30% identity.

Bacterial ferritins N-terminus is very well conserved, whereas C-terminus is not so conserved that could be due to different lengths of E-helix. The C-terminus of Hpf is projected into the inner cavity, and the E-helix is fully buried inside the protein, probably driven by buried hydrophobic interactions. These residues could have a profound effect on the integrity of the 24-mer and hydrophobic interactions.

Studies have reported that ferritin is extremely tolerant to mutations and alterations and such changes can be accommodated without disturbing the general assembly of the protein cage (Sonia Levi et al. 1989). To date, all known ferritins with three-dimensional structures of 24 structurally equivalent subunits assembled into a cage like oligomer are highly conserved (Chenyan Lv et al. 2014). At present, no atomic-level explanation of self-assembling mechanism forming a 24-mer and the role of longer E-helix in some organisms is not known. Assembly predicted to be kinetically driven is a self-sorting process that efficiently recruits pairs of subunits to yield subunit oligomers at a controlled rate. Ferritin dimers are predicted as building blocks, intermediates in nanocage self-assembly (Bernacchioni et al. 2014).

Conclusion

All the three constructs *viz.* Hpf-151^{N147}, Hpf-151 and Hpf- full^{L151A} showed part of the protein is assembled indicating that at least 151 residues are necessary for its assembly into a 24-mer. Leu151 and Leu153 are the hydrophobic residues of the E-helix, might control the acute angle between the D and E helices formed at the 4-fold axis. The Hpf-full^{L151A} mutant did not disrupt the assembly, and the purified mutant protein behaved similar to native protein. Leu151 and Leu153 are the hydrophobic residues of the E-helix, might control the acute angle between the D and E helices formed at the 4-fold axis. The Hpf-full^{L151A} mutant did not disrupt the assembly, and the purified mutant protein behaved similar to native protein.

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Figures

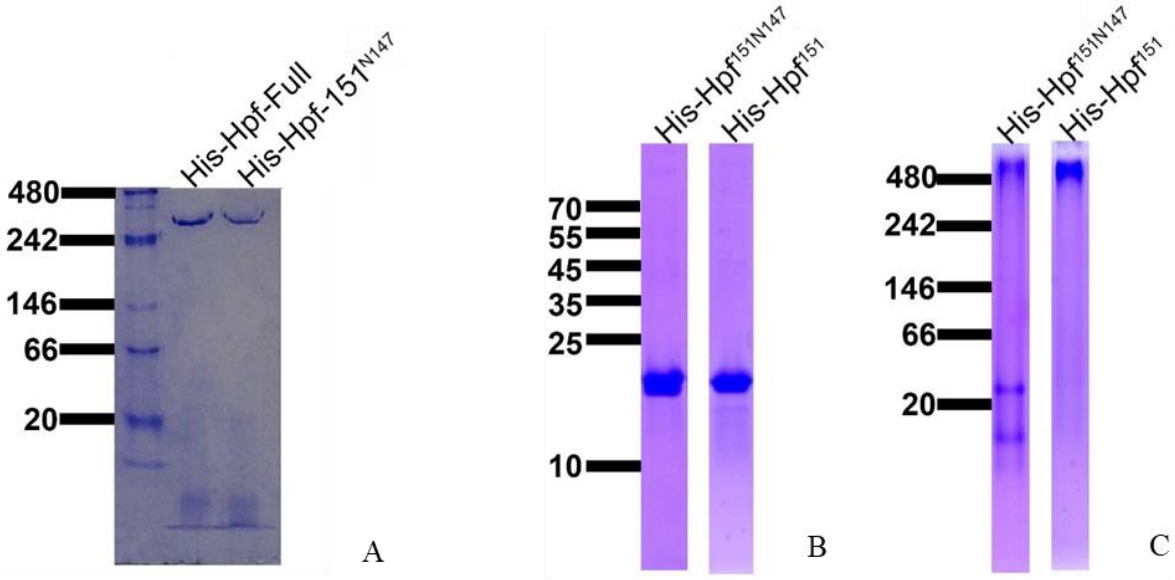

Figure 1. Native PAGE and SDS-PAGE profile showing Hpf-Full and Hpf-151

(A) Native PAGE (12.5%) showing both Hpf-full and Hpf-151^{N147}

(B) SDS-PAGE showing Hpf-151^{N147} and Hpf-151

(C) Native-PAGE showing Hpf-151^{N147} and Hpf-151

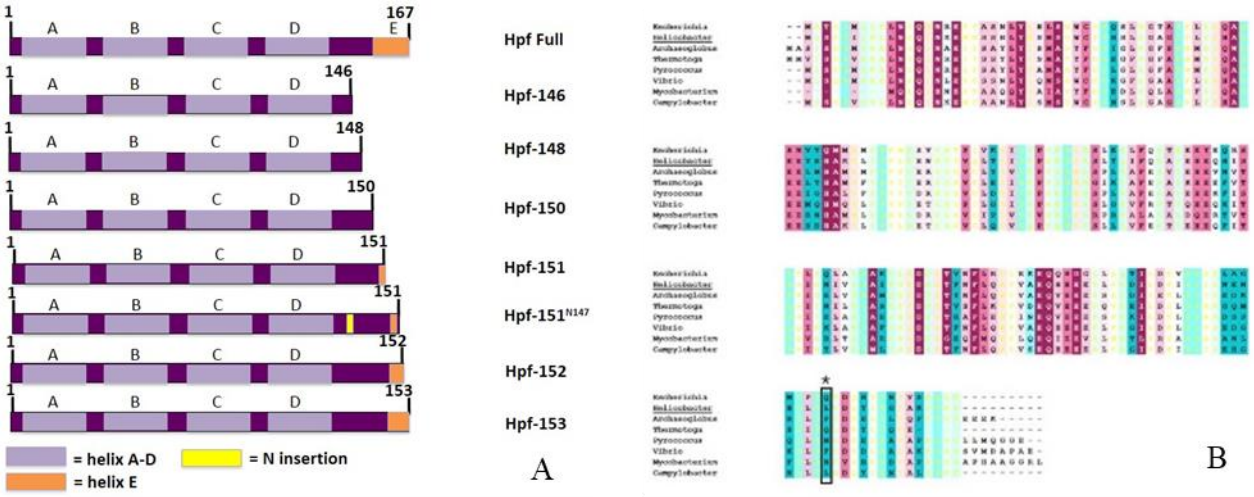

Figure 2. E-helix truncations and multiple sequence alignment of eubacterial ferritins, where Hpf L151 is highlighted

(A) Schematic depiction of Hpf constructs cloned and expressed to check the assembly.

(B) Sequence alignment using *H. pylori, E. coli, A. fulgidus, T. maritima, P. furiosus, V. Cholerae, M. tuberculosis, C. jejuni* ferritins: *C. jejuni* ferritins showed 63% identity, whereas other ferritins showed between 30-47% identity.

Table 1. List of oligonucleotides used to construct the Hpf truncations

Gene	Primer	Sequence
Hpf ^{Full}	Fwd	5′ CGGGATCCGATGTTATCAAAAGACATCATTAAGTTGCTA 3′
	Rev	5′ CCGCTCGAGTCATTAAGATTTCCTGCTTTTAGCGAT 3′
Hpf ^{146}	Fwd	5′ CGGGATCCGATGTTATCAAAAGACATCATTAAGTTGCTA 3′
	Rev	5′ CTCGAGTTACATGAATGCAGTTCTTC 3′
Hpf ^{148}	Fwd	5′ CGGGATCCGATGTTATCAAAAGACATCATTAAGTTGCTA 3′
	Rev	5′ CCGCTCGAGTTAGTTATTATTACCAATCAACTCAATTTT 3′
Hpf ^{150}	Fwd	5′ CGGGATCCGATGTTATCAAAAGACATCATTAAGTTGCTA 3′
	Rev	5′ GAATTCTTATGCGGTGTTGAATTTGTCGA 3′
Hpf ^{151}	Fwd	5′ CGGGATCCGATGTTATCAAAAGACATCATTAAGTTGCTA 3′
	Rev	5′ GAGTTACATGAATGCAGTTCTTC 3′
Hpf ^{151N147}	Fwd	5′ CGGGATCCGATGTTATCAAAAGACATCATTAAGTTGCTA 3′
	Rev	5′TCGCTTCACGAGCTGAGCAATGATCTCGGCTTG3′
Hpf ^{152}	Fwd	5′ CGGGATCCGATGTTATCAAAAGACATCATTAAGTTGCTA 3′
	Rev	5′ GGCCTCGAGTTAATACAAGCCATGGTTTTCATTA 3′
Hpf ^{153}	Fwd	5′ CGGGATCCGATGTTATCAAAAGACATCATTAAGTTGCTA 3′
	Rev	5′ GGCCTCGAGTTATAAATACAAGCCATGGTTTTCA 3′