J. Microbiol. Biotechnol. (2005), **15**(2), 254–258

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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In Vitro Formation of Protein Nanoparticle Using Recombinant Human Ferritin H and L Chains Produced from *E. coli*

RO, HYEON-SU, HYUN-KYU PARK, MIN-GON KIM, AND BONG HYUN CHUNG*

BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, P.O.Box 115, Yuseong, Daejeon 305-600, Korea

Received: March 29, 2004
Accepted: July 25, 2004

Abstract We have conducted *in vitro* reconstitution study of ferritin from its subunits FerH and FerL. For the reconstitution, FerH was produced from an expression vector construct in *Escherichia coli* and was purified from a heat-treated cell extract by using one-step column chromatography. FerL was expressed as inclusion bodies. The denatured form of FerL was obtained by a simple washing step of the inclusion bodies with 3 M urea. The reconstitution experiment was conducted with various molar ratios of urea-denatured FerH and FerL to make the ferritin nanoparticle with a controlled composition of FerH and FerL. SDS-PAGE analysis of the reconstituted ferritins revealed that the reconstitution required the presence of more than 40 molar% of FerH in the reconstitution mixture. The assembly of the subunits into the ferritin nanoparticle was confirmed by the presence of spherical particles with diameter of 10 nm by the atomic force microscopic image. Further analysis of the particles by using a transmission electron microscope revealed that the reconstituted particles exhibited different percentages of population with dense iron core. The reconstituted ferritin nanoparticles made with molar ratios of [FerH]/[FerL]=100/0 and 60/40 showed that 80 to 90% of the particles were apoferritin, devoid of iron core. On the contrary, all the particles formed with [FerH]/[FerL]=85/15 were found to contain the iron core. This suggests that although FerH can uptake iron, a minor portion of FerL, not exceeding 40% at most, is required to deposit iron inside the particle.

Key words: Ferritin, refolding, reconstitution, nanoparticle

Iron is an essential nutritional component for living organisms but the chemistry of iron at physiological conditions is not favorable to form biologically accessible iron. Moreover, the presence of excessive amount of iron is toxic to cell function [4]. For these reasons, cells require devices to store intracellular iron as a soluble, nontoxic form. The most common strategy is the use of ferritin as iron storage. Ferritin is a protein complex with a molecular weight of 450 kDa. It is a spherical complex with the external diameter of 12 nm and the internal diameter of 8 nm and consists of 24 subunits of heavy (FerH) and light (FerL) chains [5]. One molecule of ferritin stores up to 4,500 iron atoms. The molecular weights of FerH and FerL are 21 kDa and 20 kDa, respectively. The molar ratio of FerH and FerL in the ferritin is largely dependent on organs and species. For example, horse spleen ferritin consists of 85% of FerL and 15% of FerH, whereas human spleen ferritin contains more than 90% of FerL. Brain and heart contain ferritin mostly with FerH [3]. FerH subunit has a ferroxidase activity to oxidize Fe^{2+} ion into Fe^{3+} ion and is responsible for rapid uptake of iron. FerL assists in the formation of iron core and promotes the growth of Fe(III) clusters ($5Fe_2O_3{\cdot}9H_2O$) [9, 11]. Because of the functional difference between the subunits, the control of the molar ratio of FerH and FerL is one of the important factors in the reconstitution of the ferritin complex *in vitro*.

Ferritin has been developed as a device to deliver bioavailable iron for the general population, especially for women, who are frequently suffering iron deficiency. Besides the medical use, ferritin has been sought for other applications due to its spherical shape and internal cavity. The incorporation of magnetite into the apoferritin has been conducted to manufacture magnetoferritin [12, 18, 19]. Because of its magnetic property, magenetoferritin, which contains magnetite (Fe_3O_4) instead of the hydrated iron oxide, has been implied to have potential in biomedical and industrial applications, such as an information storage device and for biomedical imaging [1]. In addition, there has been efforts to generate the ferritin complexes with various ratios of FerH and FerL by coexpressing them

*Corresponding author
Phone: 82-42-860-4442; Fax: 82-42-879-8594;
E-mail: chungbh@kribb.re.kr

either by using a dual vector system or using a bicistronic expression system with single vector [8, 15]. Although the multicistronic system often results in a successful expression of proteins [14], the approaches, however, inevitably generate a heterogeneous population of ferritins with different ratios of FerH and FerL [2, 16].

In this study, we demonstrate the *in vitro* reconstitution of ferritin nanoparticles with controlled molar ratios of urea-denatured recombinant FerH and FerL subunits. The formation of spherical ferritin nanoparticles was confirmed by using transmission electron microscopy (TEM) and atomic force microscopy (AFM).

MATERIALS AND METHODS

Plasmid Construction

The genes encoding human FerH and FerL were amplified by a polymerase chain reaction (PCR) using the 5'-primer (TCGAATTCATGACGACCGCGTCCACC) and the 3'-primer (ATA CTC GAG GCT TTC ATT ATC ACT) for FerH and the 5'-primer (TCG AAT TCA TGA GCT CCC AGA TTC GT) and the 3'-primer (ATA CTC GAG GTC GTG CTT GAG AGT) for FerL from a human cDNA library. Thirty cycles of PCR were performed by using a Pfu DNA polymerase. Each cycle consisted of the following three steps: 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min. The resulting DNA product was purified with a PCR extraction kit (Qiagen, U.S.A.) and digested with the restriction enzymes *Eco*RI and *Xho*I. The resulting DNA fragment was ligated into the *Eco*RI and *Xho*I sites of the pET22b vector (Novagen, U.S.A.), using a DNA ligation kit (Takara, Japan).

Protein Expression and Preparation of Cell Extract

The expression vectors were transformed into *E. coli* BL21(DE3) competent cells. The resulting recombinant cells were grown in 250 ml of LB medium (yeast extract 5 g/l, tryptone 10 g/l, NaCl 5 g/l), containing ampicillin (100 μg/ml), at 30°C. Production of the human ferritin was induced by addition of 1 mM IPTG when optical density at 600 nm of the culture broth reached 0.6. After 4 additional h of culture, the cells were harvested by centrifugation (6,000 rpm, 15 min). The harvested cells were washed twice with 10 ml of phosphate-buffered saline (PBS) (NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l, KH_2PO_4 0.24 g/l, pH 7.4). The cells were resuspended in 10 ml of 50 mM Tris-HCl (pH 8.0) and destroyed by sonication. The soluble and insoluble fractions of the sonified suspension were obtained by centrifugation (13,000 rpm, 15 min). The insoluble fraction was dissolved in 50 mM Tris-HCl (pH 8.0) containing 8 M urea. The proteins were analyzed by using 12% SDS-polyacrylamide gel electrophoresis and visualized by Coomassie brilliant blue R-250 staining.

Purification and Preparation of Denatured Ferritin

FerH was prepared according to the method previously reported [6, 7, 10, 17]. In brief, the crude extract, containing FerH, was incubated at 70°C for 20 min. The supernatant was loaded onto a DEAE-sepharose ion-exchange column. The bound protein was eluted with a linear gradient of NaCl (0- 1 M) in 20 mM Tris-HCl (pH 8.0). For *in vitro* reconstitution, the purified FerH was denatured with a denaturation buffer [50 mM Tris-HCl (pH 8.0), 1.0 mM EDTA, 8 M Urea, 100 mM β-Mercaptoethanol]. FerL was produced as an insoluble form. The insoluble fraction of the sonified cells, containing FerL, was collected and washed with washing buffer [50 mM Tris-HCl (pH 8.0), 1.0 mM EDTA, 3 M urea]. The resulting pellet was dissolved in the denaturation buffer.

TEM and AFM Image Analyses

An energy filtering transmission electron microscopy (EF-TEM, Carl-Zeiss Co., EM 912 Omega, German) was conducted at Korea Basic Science Institute (KBSI). The sample was negatively stained with 0.2% ammonium molybdate prior to analysis. An atomic force microscopy (AFM, Nanonics Co., Israel) was conducted with a 10 nm cantilever, made of glass fiber. The AFM substrate was mica. The protein was adsorbed on the surface of the mica in a 50 mM sodium phosphate buffer (pH 8.0) containing 0.2 M NaCl. The surface was washed with deionized water and dried with a gentle blowing of nitrogen gas.

RESULTS AND DISCUSSION

Expression and Purification of FerH and FerL

Figure 1A shows the expression of recombinant FerH and FerL in *E. coli*. FerH was mostly expressed as a soluble form, whereas the major portion of FerL was insoluble. The heat-treated crude extract, containing FerH, was loaded onto a DEAE column and eluted with a linear gradient of 0 M NaCl to 1 M NaCl (Fig. 1B). FerH was eluted as near homogeneity at 0.3 M NaCl (Fig. 2, inset). The final recovery yield was more than 80%. Since FerL was expressed as insoluble inclusion bodies, the purification of FerL was rather simple. The FerL-containing sonified cells were centrifuged, and the pellet was collected. The pellet was washed with washing buffer containing 3 M urea. The washed pellet was dissolved in 50 mM Tris-HCl (pH 8.0) buffer containing 8 M urea. The purity of the FerL was more than 90% (Fig. 1C).

In Vitro Reconstitution of Ferritin

In order to reconstitute the ferritin nanoparticle with its subunits of FerH and FerL, both subunits were dissolved in 50 mM Tris-HCl (pH 8.0) buffer, containing 1 mM EDTA, 8 M urea, and 100 mM 2-mercaptoethanol. The denatured

subunits were mixed together at various ratios. Total concentration of [FerH+FerL] was 20 μM, and the reaction volume was 0.5 ml. The resulting mixtures were dialyzed against a dialysis buffer [50 mM sodium phosphate (pH 7.4), 0.15 M NaCl, 1 mM ferrous ammonium sulfate] for 6 hours. The dialyzed samples were centrifuged to remove proteins that were not incorporated into the ferritin complex. As shown in Fig. 2A, the ratios of input proteins (FerH and FerL) was [FerH]/[FerL]=100/0, 90/10, 85/15, 70/30, 60/40, 40/60, 30/70, and 0/100. The supernatants of the dialyzed samples that contained the reassembled ferritin nanoparticles were analyzed by SDS-PAGE (Fig. 2B). The analysis revealed that FerL alone or the mixture with high concentration of FerL could not be assembled into the ferritin nanoparticle (Fig. 2B, see the [FerH]/[FerL]=30/70 and 0/100). To make ferritin nanoparticle by *in vitro* reconstitution, it became evident that the reaction mixture should contain more than 40% of FerH subunit. In order to verify the reason why FerL alone could not form the ferritin complex, we analyzed the pellets of dialyzed samples. As shown in Fig. 2C, most of the input FerL was found in the aggregated pellet, especially in the mixture without FerH or in the mixture with a low ratio of FerH.

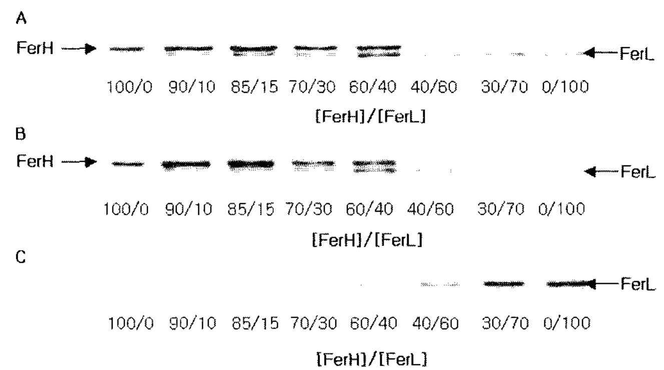

Fig. 2. *In vitro* reconstitution of the ferritin nanoparticle.
A) Inputs of the denatured FerH and FerL to the reconstitution reaction mixture. Total concentration of [FerH+FerL] was 20 μM, and the reaction volume was 0.5 ml. B) and C) Outputs after the reconstitution experiment. The reconstituted samples were centrifuged. The supernatants (B) and the pellet (C) of the centrifuged samples were analyzed. The numbers under the gel indicate the ratio of FerH and FerL.

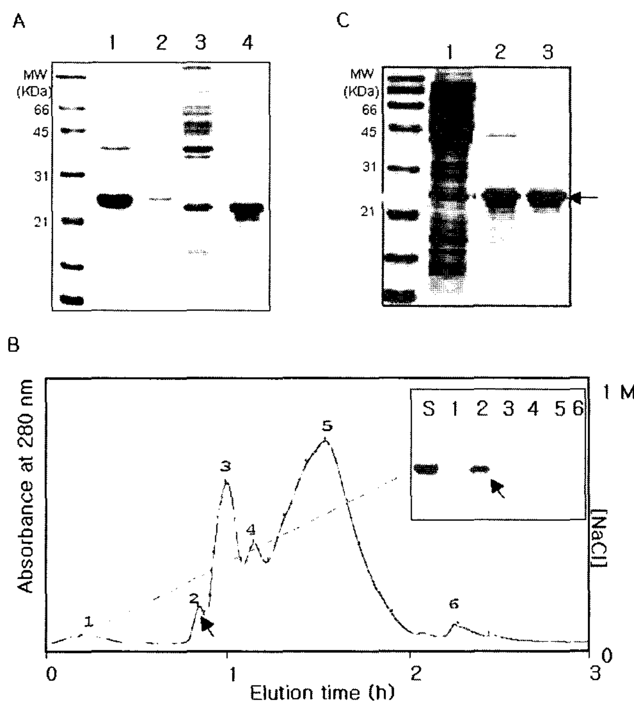

Fig. 1. Expression and purification of human ferritin in recombinant *E. coli*.
A) Expressions of FerH and FerL. Lane 1: FerH in soluble fraction; lane 2: FerH in insoluble fraction; lane 3: FerL in soluble fraction; lane 4: FerL in insoluble fraction. B) DEAE ion-exchange column chromatography of FerH. The numbers on the curve and the SDS-PAGE gel image are collected fraction numbers. The arrow indicates the FerH-containing fraction. The lane S in the inset is the heat-treated crude extract. C) SDS-PAGE analysis of FerL. Lane 1: the cell pellet after sonication; lane 2: the pellet washed with 3 M urea; lane 3: FerL dissolved in 8 M urea. The arrow indicates FerL.

Atomic Force Microscope (AFM) and Transmission Electron Microscope (TEM) Analyses of the Reconstituted Ferritin

In order to verify if the reconstituted ferritin nanoparticle was actually assembled into a spherical shell, we analyzed

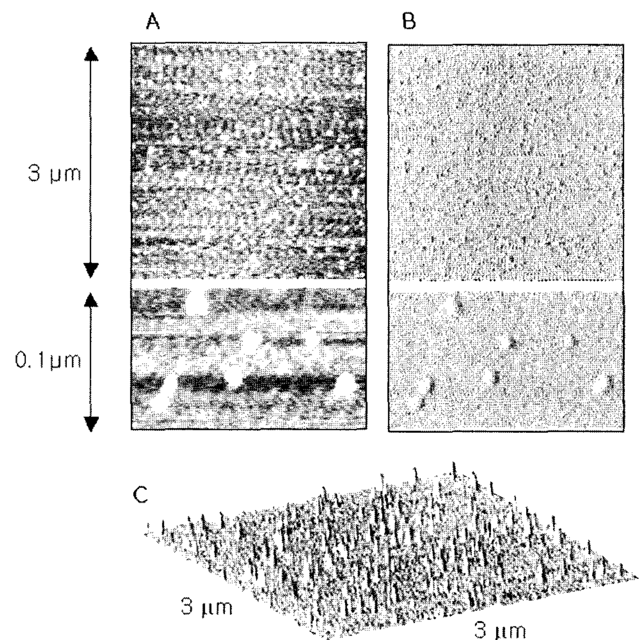

Fig. 3. Atomic force microscopic analysis of the reconstituted ferritin.
The ferritin nanoparticle with [FerH]/[FerL]=85/15 was adsorbed onto the mica surface. The surface was scanned by using a cantilever with 10 nm resolution. Height (A), amplitude (B), and 3D images (C) are shown. The numbers indicate the scale of the image.

the particle by using an atomic force microscope (AFM) [13]. The ferritin particle with [FerH]/[FerL]=85/15 was first adsorbed onto the surface of mica, and then the surface was scanned with a glass-fiber cantilever with the resolution of 10 nm. The scan area was 3 μm×3 μm, as shown in Fig. 3. The scanned image revealed that the particle was well adsorbed onto the surface and was a spherical shape with approximate diameter of 10 nm and height of 10 nm. To obtain better information on the particle, we subsequently analyzed the ferritin nanoparticles with a transmission microscope (TEM). The samples used for this analysis were the ferritin nanoparticle with [FerH]/[FerL]= 100/0 (Fig. 4A), 85/15 (Fig. 4B), 60/40 (Fig. 4C), and a commercially available horse spleen ferritin (Fig. 4D) as a control experiment. The TEM images revealed that all samples formed spherical ferritin nanoparticles with the approximate external diameter of 10 nm. A close look of the image revealed that some particles contained a dense core inside the shell. This type of dense core inside the shell had been reported to be an iron core [18]. To verify the effect of subunit composition of the ferritin nanoparticle on the deposition of iron, we calculated the number of the iron core-containing particles from the total number of the particle. The result is summarized in Table 1. A commercially available horse spleen ferritin, which consisted of 15% of horse FerH and 85% of horse FerL, showed that 70% of the total particles contained the iron core. Less than 10 to 20% of the reconstituted human ferritin particles in the

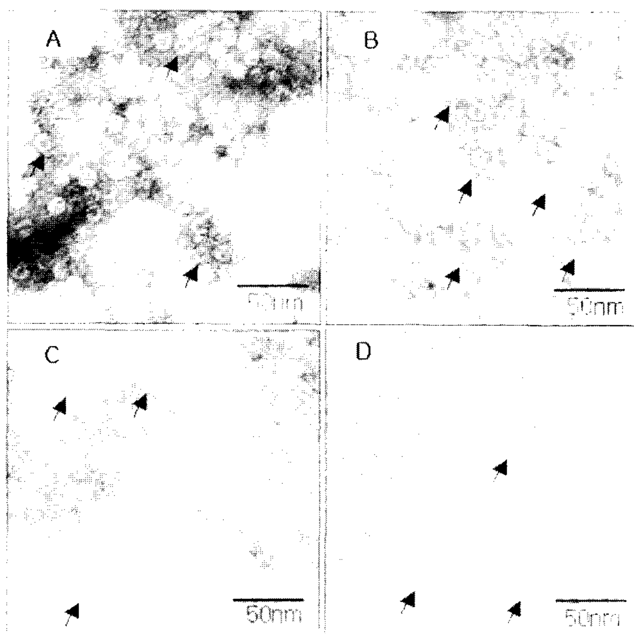

Fig. 4. Transmission electron microscopic analysis of the reconstituted ferritin.
A) [FerH]/[FerL]=100/0. B) [FerH]/[FerL]=85/15. C) [FerH]/[FerL]=60/40. D) Horse spleen ferritin. The arrows indicate the ferritin particles with a dense iron core.

Table 1. Effect of ratio of FerH and FerL in ferritin on the formation of iron core.

	A	B	C	D*
[FerH]:[FerL]	100:0	85:15	60:40	15:85
No. of ferritin with iron core	108	121	89	173
Total No. of ferritin counted	22	121	9	121
% of ferritin with iron core	20	100	10	70

*Commercially available horse spleen ferritin.

population with [FerH]/[FerL]=100/0 and 60/40 contained the iron core. Surprisingly, 100% of the reconstituted particles with [FerH]/[FerL]=85/15 had the iron core. This suggested that the reconstituted ferritin nanoparticle requires a minor portion of FerL subunit to deposit iron inside the particle. The reason of why a higher percentage of the population from the commercial horse spleen ferritin contained the iron core, despite a high ratio of FerL, is not clear at present. It may reflect a subtle structural difference between the reconstituted ferritin and the naturally occurring one.

Acknowledgments

This research was supported by grants from the KRIBB Initiative Research Program and Korea Science and Engineering Foundation through the Center for Advanced Bioseparation Technology (BSEP).

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