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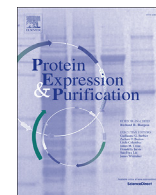

Expression, purification, and characterization of recombinant human L-chain ferritin

Wenyan Zou, Xiaoyu Liu, Xi Zhao, Jie Wang, Dianhua Chen, Jiahuang Li, Lina Ji*, Zichun Hua**

State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, 163 Xianlin Avenue, Nanjing 210046, Jiangsu, PR China

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ABSTRACT

Ferritins form nanocage architectures and demonstrate their potential to serve as functional nanomaterials with potential applications in medical imaging and therapy. In our study, the cDNA of human L-chain ferritin was cloned into plasmid pET-28a for its overexpression in *Escherichia coli*. However, the recombinant human L-chain ferritin (rLF) was prone to form inclusion bodies. Molecular chaperones were co-expressed with rLF to facilitate its correct folding. Our results showed that the solubility of rLF was increased about 3-fold in the presence of molecular chaperones, including GroEL, GroES and trigger factor. Taking advantage of its N-terminal His-tag, rLF was then purified with Ni-affinity chromatography. With a yield of 10 mg/L from bacterial culture, the purified rLF was analyzed by circular dichroism spectrometry for its secondary structure. Furthermore, the rLF nanocages were characterized using dynamic light scattering and transmission electron microscopy.

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1. Introduction

Isolated from horse spleen for the first time in 1937, ferritins are a family of proteins in charge of iron storage, iron release [1] and detoxification [2]. It is known that the 24 subunits of ferritins self-assemble into spherical cage architectures with protein shells and iron cores. Subunit analysis reveals that mammalian ferritins are composed of two different subunits known as H- and L-chains [3]. Each subunit forms a bundle of four long and a fifth short α-helix with a short non-helical extension [4,5]. Two types of subunits in different proportions compose a complex of 24 subunits in human tissues and organs [6,7]. In addition, the ratio of both H- and L-chains would vary depending on the pathological changes [7,8]. With an external size of 12–13 nm and an interior cavity of 7–8 nm in diameter [6,9], the hollow protein shell of ferritin is capable of hosting up to 4500 iron atoms [6,10]. Its inner cavity has been exploited as a reaction chamber for the template synthesis of nanoparticles with a well-defined size and a narrow size distribution [11,12]. A recombinant human H-chain ferritin-iron oxide nano-composite has been investigated as a magnetic resonance contrast agent [13]. Furthermore, ferritin nanocages have been used to transport drugs to different targets through either genetic modification or chemical conjugation [14–16].

In 2006, recombinant human L-chain ferritin (rLF) was revealed to be composed of 24 subunits, with each monomer composed of a 17-residue four-helix bundle, and a fifth helix linked at the C-terminal end [17]. Although H- and L-chains share 53% identical amino acid sequences and have similar three-dimensional structures [6,18], they perform different functions during the process of iron deposition [19]. H-chain contains a catalytic ferroxidase site that catalyzes the oxidation of Fe (II) to Fe (III) [9,20]. In contrast to H-chain, L-chain shows a capacity to induce iron mineralization with higher efficiency [21]. L-chain provides efficient nucleation sites for iron, and accelerates the development of the iron core [22–24]. The most recent study on the specific functions of L-chain showed that the electrons released during iron-oxidation were transported across the ferritin cages specifically through the L-chain and the inverted electron transport through the L-chain also accelerated the demineralization of ferritin [25]. Besides, a new ferritin

Abbreviations: CD, circular dichroism; DLS, dynamic light scattering; *E. coli*, *Escherichia coli*; HSF, horse spleen ferritin; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate buffered saline; rLF, recombinant human L-chain ferritin; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TET, tetracyclines; TF, trigger factor.

* Corresponding author.
** Corresponding author.
E-mail addresses: jilina@nju.edu.cn (L. Ji), huazc@nju.edu.cn (Z. Hua).

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receptor specific to L-chain was also identified as Scara5 that binds ferritin and then stimulates its endocytosis from the cell surface with consequent iron delivery [26,27]. Therefore, it is necessary to investigate the potential applications of H- and L-chains according to their distinct properties. However, the applications of L-chain are quite limited, partly because it forms inclusion bodies in *Escherichia coli* (*E. coli*) and its yield is reported to as low as 2–5 mg/L from bacterial culture [28]. In this study, we significantly improve the soluble yield of recombinant L-chain ferritin.

Here we report a study to obtain rLF with high purity from *E. coli*. The yield of rLF is improved to 10 mg/L from bacterial culture with purity up to 96%. Then the rLF was characterized by circular dichroism (CD) spectrometry for its secondary structure. Moreover, the rLF cages were analyzed by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

2. Materials and methods

2.1. Vectors,strains and chemicals

Plasmid pET-28a, plasmid pG-Tf2 and host strains *E. coli* BL21 (DE3) were maintained in our laboratory. TRIzol reagent was from Invitrogen. PrimeScript RT Reagent Kit, phusion DNA polymerase, dNTP mix, restriction endonucleases and T4 DNA ligase were obtained from TaKaRa. Plasmid isolation kit was purchased from Shanghai Bocai Company. The isopropyl-β-D-thiogalactopyranoside (IPTG), tetracyclines (TET) and phosphotungstic were purchased from Sangon Biotech. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein standard was obtained from Thermo Scientific. Horse spleen ferritin (HSF) was obtained from Sigma–Aldrich. BCA protein assay kit was purchased from CWBio. The Ni^{2+}-Sepharose 6 Fast Flow was obtained from GE Healthcare.

2.2. Construction of plasmid pET-28a-rLF

Total RNA was extracted by TRIzol reagent from human lung adenocarcinoma A549 cells, and used for the reverse transcription reaction by PrimeScript RT Reagent Kit. Then the product of the reverse transcription reaction was used for amplification of rLF cDNA with primers designed based on its sequence in Genbank (M10119.1). For subsequent cloning into the bacterial expression plasmid pET-28a, the primers introduced restriction endonuclease cleavage sites for BamHI and XhoI, respectively. The constructed plasmid pET-28a-rLF was verified by DNA sequencing.

2.3. Expression of rLF alone

The BL21 transformed with pET-28a-rLF was cultured in LB medium containing 50 mg/L kanamycin. The LB medium was shaken at 37 °C until the OD_{600} reached 0.8, then IPTG was added to a final concentration of 1 mM to induce rLF expression. The BL21 cells were further cultured for 15 h at 20 °C or 5 h at 37 °C, and collected by centrifugation at 5000 rpm for 10 min at 4 °C.

2.4. Co-expression of rLFand molecular chaperones

The BL21 transformed with pET-28a-rLF (kanamycin-resistant) and pG-Tf2 (chloramphenicol-resistant) was cultured in LB medium containing kanamycin (50 mg/L) and chloramphenicol (34 mg/L) at 37 °C, until the OD_{600} reached 0.8. To induce expression of molecular chaperones, TET was then added at a final concentration of 20 ng/L. After 30 min, 1 mM IPTG was added to trigger the expression of rLF. Then BL21 cells were further cultured for 15 h at 20 °C or 5 h at 37 °C, and collected by centrifugation.

2.5. Purification of rLF

BL21 cells co-expressing rLF and molecular chaperones were harvested by centrifugation, and resuspended in 10 mM phosphate buffered saline (PBS, pH7.4) for sonication on ice. The cell lysate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was collected. After the addition of imidazole to a final concentration of 10 mM, the supernatant was applied onto a 10-ml Ni^{2+}-Sepharose 6 Fast Flow column pre-equilibrated with 10 mM PBS containing 10 mM imidazole. The column was then washed with 10 and 100 mM imidazole to remove nonspecifically bound proteins, and eluted with 250 and 500 mM imidazole. The eluted fraction of rLF protein was dialyzed overnight in 10 mM PBS to remove imidazole. The purity of rLF was analyzed by 12% SDS-PAGE. The final protein concentration was measured by BCA protein assay kit to calculate its yield from bacterial culture.

2.6. Analysis of purified protein

The purified rLF was prepared as described above. The ferritin from horse spleen (HSF) was regarded as a reference. Far-UV CD spectra of rLF and the HSF were measured by a JASCO J-810 spectropolarimeter at 25 °C. The protein samples were diluted to 5 μg/mL, and transferred to a 0.05-cm path length quartz cuvette. For each spectrum, an average of three scans was obtained at a scan rate of 50 nm/min, and the data were presented as relative ellipticity (mdeg) in the range of 200–250 nm.

Each TEM sample was prepared by depositing a drop of protein solution (1 mg/mL) on the surface of a copper net coated with carbon. After adsorption for 5 min, the samples were stained by 2% phosphotungstic acid for 1–2 min. Images were obtained using a JEOL TEM (JEM-2100, Japan) at 200 kV. Every protein sample was imaged for at least three times independently and each sample was observed in more than five regions to avoid experimental errors.

The size of the protein nanoparticles (1 mg/mL) was measured using DLS (Malvern Zetasizer Nano S90; UK), and the mean value of triplicate measurements was adopted for analysis.

3. Results

3.1. Identification of the optimized conditions for rLF expression

It is common that recombinant proteins form inclusion bodies when they are overexpressed in prokaryotic expression system. Optimization of temperature for protein expression is suggested to be a feasible way to improve the solubility of recombinant proteins [29]. Here the expression of rLF was tested at 20 °C and 37 °C, respectively. After sonication and centrifugation, BL21 cell lysate was separated as supernatant and pellet fractions. Then the levels of rLF in cell lysate, supernatant and pellet fractions were analyzed by SDS-PAGE. As shown in Fig. 1, majority of rLF was in pellet fractions at both 20 °C (lane 3) and 37 °C (lane 6). The amount of soluble rLF in supernatant was not changed significantly when the temperature was lowered from 37 °C (lane 5) to 20 °C (lane 2).

Molecular chaperones are known to facilitate proteins folding [29,30]. Co-expression of molecular chaperones with recombinant proteins improves the solubility of recombinant proteins [31]. *E. coli* trigger factor (TF) is a new chaperone-like factor, which plays synergistic roles with molecular chaperones GroEL-GroES to facilitate protein folding and enhance production of active proteins. When induced by TET, plasmid pG-Tf2 can synchronously express molecular chaperones, including GroES (10 kDa), GroEL (60 kDa) and Tf (56 kDa) [32,33]. Therefore, pG-Tf2 was transformed into BL21 together with pET-28a-rLF to investigate whether the molecular chaperones can increase rLF expression in soluble form.

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