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Improved Coexpression and Multiassembly Properties of Recombinant Human Ferritin Subunits in *Escherichia coli*

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Human heavy chain (H-) and light chain (L-) ferritins were amplified from a human cDNA library. Each ferritin gene was inserted downstream of the T7 promoter of bacterial expression vectors, and two types of coexpression vectors were constructed. The expression levels of recombinant ferritins ranged about 26–36% of whole-cell protein. H-ferritin exhibited a lower expression ratio compared with L-ferritin, by a coexpression system. However, the coexpression of HL-ferritins was significantly increased above the expression ratio of H-ferritin by cultivation without IPTG induction overnight. Purified recombinant H-, L-, HL-, and LH-ferritins were shown to be homo- and heteropolymeric high molecular complexes and it was indicated that their assembled subunits would be able to work functionally in the cell. Thus, these results indicate an improvement in the expression strategy of H-ferritin for heteropolymeric production and studies of ferritin assembly in *Escherichia coli*.

Keywords: Human ferritin, coexpression, multiassembly

Ferritin is an iron storage protein that plays an important role in iron metabolism. Ferritin is found in mammalian, plant, and bacterial cells. They store up to 4,500 iron atoms in a central cavity in the form of a hydrous ferric oxide mineral core [5]. In mammals, two types of subunit, H (heavy) and L (light) polypeptides, have over 50% amino acid sequence homology, and molecules are found in varying ratios in ferritin from different tissues [10, 30]. The H-ferritin can induce a rapid oxidation of iron from Fe (II) to Fe (III) owing to the presence of a ferroxidase center located in the inner portion of the subunit fold [19, 20, 28] cavity attributed to the presence of a carboxyl group exposed on the cavity surface [21, 28]. *In vivo*, two main subunit types are found in various coassembled molecules with various proportions [3]. Natural assembled ferritins are homopolymers and heteropolymers, whereas mostly heteropolymeric molecules are found in various tissues. The heteropolymer ferritin maintains the biochemical properties of H-, L-ferritins and consequently is more efficient in iron oxidation and mineralization [22]. So far, a variety of recombinant ferritins in the mouse, rat, bovine, tadpole, dog, horse, and human have been reported and some have been expressed in *E. coli*, insect cells, and yeasts [3, 8, 12, 15, 16, 24–27]. Although recombinant H- and L-ferritins have been expressed in both prokaryotic and eukaryotic cells, H-ferritin has been demonstrated to suppress cell growth and show low expression levels of H-ferritin from coexpression systems. In order to overcome the suppressed expression of H-ferritin in coexpression, rifampicin has been added to the media and an inducible expression system has been used [6]. However, unbalanced coexpression of H- and L-ferritins has been a critical problem in the biochemical study of ferritin. Furthermore, construction of a recombinant ferritin assembly has only been attempted *in vitro*.

In this study, recombinant human HL- and LH-ferritins were cloned and expressed in *E. coli* in order to study their uniform expression and to characterize heteropolymeric ferritin assembly.

Materials and Methods

Bacterial Strains, Plasmid, and Medium

E. coli DH5α was used for transformation and construction of plasmids. *E. coli* BL21 (DE3) was used for expression of the H-, L-, HL-, and LH-ferritin genes. The expression vector pET5b was obtained from Novagen (Madison, WI, U.S.A.). *E. coli* cells containing plasmid were grown in LB medium supplemented with 50 μg/ml ampicillin.

Reverse Transcription PCR

For obtaining ferritin genes, the cDNA for human H- and L-subunits were prepared by reverse transcription PCR from human

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heart mRNA and liver mRNA (Clontech, Palo Alto, CA, U.S.A.), respectively. Reverse transcription was performed at 37°C for 1 h (Takara, Japan). Ferritin specific primers were used to amplify the human H- and L-ferritin cDNA. Primers were synthesized based on the human ferritin mRNA sequences from GenBank. To enable the cloning of amplified fragments, the primers were modified by insertion of a linker adapter, which was created by NdeI and BamHI restriction sites. The primer sequences were as follows (Bioneer, Taejeon, Korea): 5'-primer for H-ferritin: 5'-GCGCCATATGA-CGACCGCGTCCACCTCGCAGGTGCGCCA-3'; 3'-primer for H-ferritin: 5'-GCGCGGATCCTTAGCTTTCATTATCACTGTCTCCC-AGGGT-3'; 5'-primer for L-ferritin: 5'-CCGGCATATGAGCTCCC-AGATTCGTCAGAATTATTCCAC-3'; and 3'-primer for L-ferritin: 5'-GCGCGGATCCTTAGTCGTGCTTGAGAGTGAGCCTTTCGAA-3'. *Taq* polymerase (Roche, Germany) was used and PCR conditions consisted of an initial denaturation step at 94°C for 5 min, 30 cycles of 94°C for 1 min, 49°C for 1 min, and 73°C for 1 min, and a final extension step at 73°C for 5 min in a thermocycler (MJ Research, Waltham, MA, U.S.A.).

Construction of H-, L-, and Co-Ferritin Expression Vector

PCR products and vector pET5b were digested with NdeI and BamHI at 37°C. These digested fragments were ligated using T4 DNA ligase overnight at 14°C. Finally, expression vectors of pEH-FE and pEL-FE were constructed, which do not involve the polyhistidine fusion system. The coexpression vector was constructed by isolating the entire expression cassette of pEH-FE and pEL-FE using AvaI and SspI digestion. The resulting fragments were ligated to recipient pET5b constructs containing H- or L-ferritins that had been digested with AvaI and NruI. Two types of coexpression vector were prepared with functional order, one with an H+L coding region orientation and the other with an L+H coding region orientation. DNA sequencing was performed using an ABI3100 automated sequencer, using a BigDye terminator kit (PE Applied Biosystems, Foster City, CA, U.S.A.) [13]. Finally, coexpression vectors of pEHL-FE and pELH-FE were constructed. The diagram of the expression vectors is shown in Fig. 1.

Transformation and Expression of Recombinant Human Ferritins

The plasmids encoding human H-, L-, H+L, and L+H ferritins were transformed into *E. coli* BL21(DE3) (Novagen, Madison, WI, U.S.A.). The transformants were selected and grown with shaking at 37°C overnight in LB broth (5 ml) with 50 μg/ml ampicillin. In batch culture expression, the saturated cells were diluted into the same medium (50 ml) to a ratio of 1/200. When the cells were grown to an absorbance of 0.4 at 600 nm, the expression of ferritins was induced by adding 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h [11]. The cells were harvested for analysis of ferritin expression. For continuous culture expression, the cells were cultured overnight without adding IPTG. After incubation for 22 h, cells were collected and disrupted (2× SDS lysis solution) for determining ferritin expression.

Protein Electrophoresis and Western Blots

For the determination of ferritin expression, cell extracts were resolved on a 13% (w/v) SDS-PAGE gel and stained with Coomassie

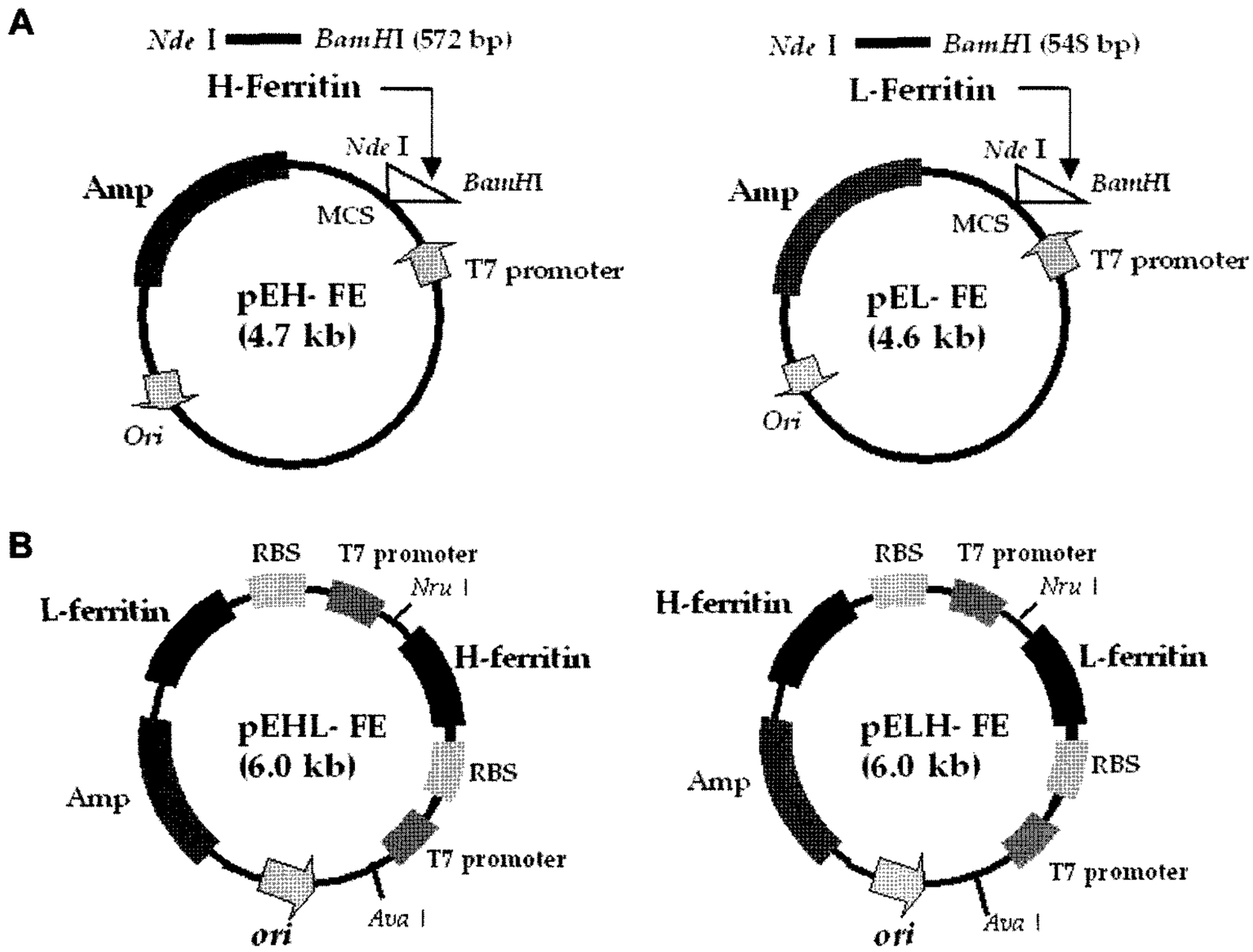

Fig. 1. Construction scheme for coexpression vectors containing human H- and L-ferritin genes.
A. Human H- and L-ferritin cDNA regions were inserted into vectors that contained unique NdeI and BamHI restriction enzyme sites. **B**. The expression cassette regions of pEH-FE and pEL-FE were isolated using AvaI and SspI digestion. Isolated fragments were inserted into separate expression vectors that contained AvaI and NruI restriction sites.

brilliant blue. The expression ratios of ferritins were determined by image scanning densitometry (Bioneer, Taejeon, Korea). For the detection of ferritin, proteins were blotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.). The nitrocellulose membrane was incubated with blocking solution containing 5% (w/v) nonfat dry milk for 1 h and washed with PBST buffer (0.9% NaCl in 10 mM sodium phosphate, 0.05% Tween-20, pH 7.4). The human ferritin specific monoclonal antibody was added as a primary antibody for 1 h. Consequently, anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody for 1 h. The membrane was washed with PBS (0.9% NaCl in 10 mM sodium phosphate buffer, pH 7.4) after primary and secondary antibody incubation. An ECL kit was used to detect the signal specific for human H- and L-ferritins.

Determination of Expression Ratio and Subunit Composition

Homo- and heteropolymer ferritins were resolved by SDS-PAGE into H and L subunits. The overexpressed ferritins were confirmed in *E. coli* by Western blotting. The expression ratios and the subunit composition of these proteins were determined by image scanning densitometry.

Purification of Recombinant Human Ferritins

After expression, the cells were collected for purification. The cell pellets were sonicated at 4°C, and the soluble homogenates were heated at 70°C for 10 min [3, 20]. Unstable proteins were removed by centrifugation and ammonium sulfate was added up to 80% saturation. Precipitated proteins were dialyzed at 4°C. After dialysis in 20 mM Tris-HCl (pH 7.4) and 50 mM NaCl, the sample was loaded onto a Mono-Q column of an FPLC system (Pharmacia, Uppsala, Sweden). The column was eluted with a 0–500 mM NaCl gradient in 20 mM Tris-HCl (pH 7.4). The fractions containing ferritin were pooled and concentrated with ammonium sulfate and dialyzed in buffer as above. The sample was loaded onto a Superose 12 column (Pharmacia, Uppsala, Sweden) of the FPLC system. Purified ferritins were finally concentrated with a Centricon 10 membrane (Millipore, Billerica, MA, U.S.A.). The protein content was determined with a protein assay kit (Bio-Rad, Munich, Germany) based on the Bradford method [23].

Nondenaturation Protein Electrophoresis

Native-PAGE and iron staining were performed for determination of ferritin assembly [8, 18]. The protein was subjected to 7.5% native-PAGE conditions and stained with Coomassie brilliant blue. For active staining of recombinant ferritins, iron staining was performed with a 2% $K_4Fe(CN)_6$, and 2% HCl mixture (1:1, v/v).

Transmission Electron Microscopy

Different types of purified recombinant ferritin were prepared for transmission electron microscopy (TEM) as described previously [17]. Samples were negatively stained with a 1% (w/w) solution of uranyl acetate. Images of electron diffraction patterns were recorded in a JEOL 1200EX electron microscope operating at 100 kV. The length of the calibrated camera was 80 cm. The particle size of ferritin was determined by a scale bar from enlarged photomicrographs [29].

Gel Permeation Chromatography

For the calculation of molecular mass, purified H-, L-, HL-, and LH-ferritins were used. Molecular masses of the ferritins were determined *via* gel filtration on a Superose12 column [2]. Standard proteins were thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobulin (17 kDa), and vitamin B-12 (1.35 kDa). The sample solution of purified ferritin was injected and eluted with Tris-HCl (pH 7.4) buffer at a flow rate of 0.5 ml/min at room temperature. The elution and void volumes were estimated automatically by a FPLC program. The equation for estimating the molecular mass was derived from plots of Ve/Vo against log MW of the standards.

Results and Discussion

Expression of Recombinant Human Ferritins in *E. coli*

In order to express ferritin of various subunits *in vivo,* the four types of recombinant human ferritin (H, L, HL, and LH) were expressed in *E. coli* by using a T7 promoter. The

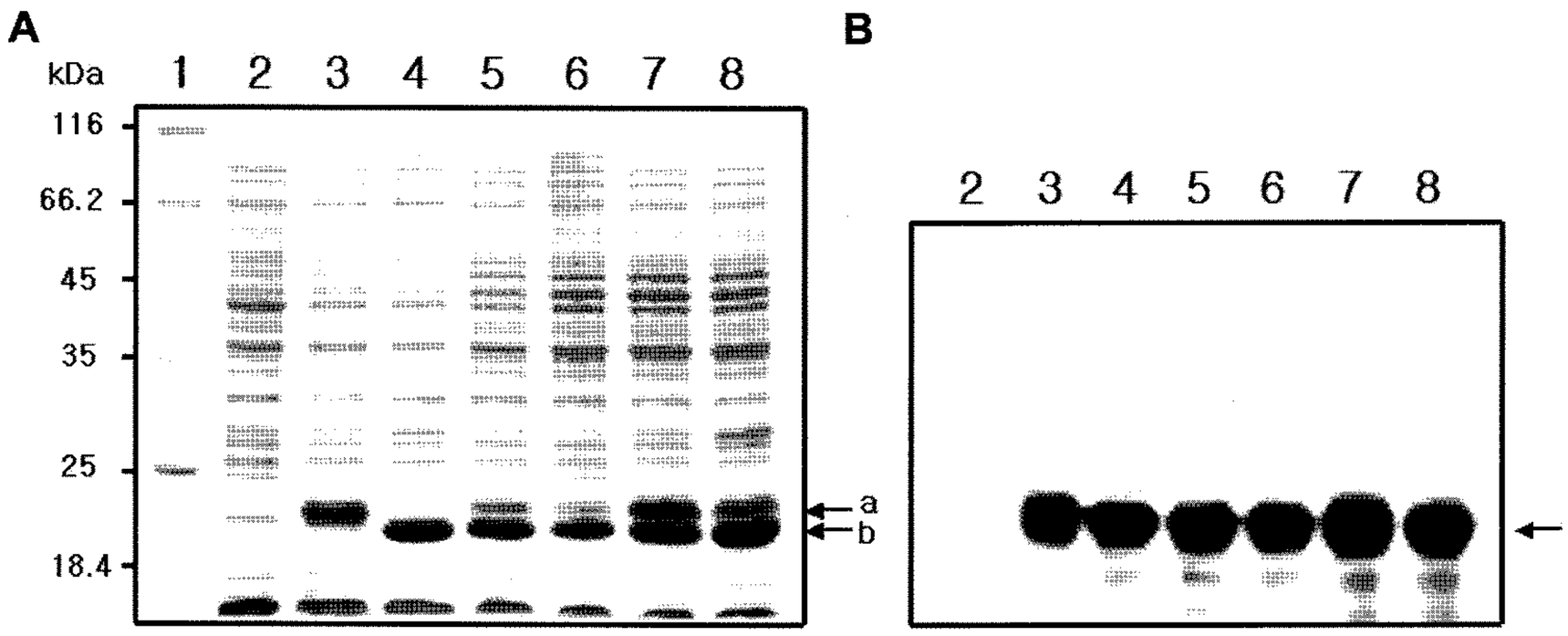

Fig. 2. SDS-PAGE analysis of recombinant human ferritins.

A. Cell extracts of IPTG induction and overnight cultivation samples were loaded onto a 13% SDS-PAGE gel and stained with Coomassie blue. **B**. Western blot analysis of recombinant human ferritins. Lane 1, protein standard maker; lane 2, negative transformant; lanes 3 and 4, overexpressed H- and L-ferritins in *E. coli* with IPTG; lanes 5 and 6, overexpressed HL- and LH-ferritins with IPTG; lanes 7 and 8, overexpressed HL- and LH-ferritins by overnight cultivation without IPTG. The arrows indicate ferritins (a: H-ferritin; b: L-ferritin).

addition of IPTG (0.4 mM) induced the ferritin synthesis for 4 h. The overexpressed standard H- and L-ferritins with apparent molecular masses of about 19 and 21 kDa were observed in SDS-PAGE and Western blots (Fig. 2). With image scanning densitometry, the expression levels of H- and L-ferritin proteins ranged between 26% and 36% of whole-cell proteins. The expression levels of HL- and LH-ferritins were about 28%. From this experiment, productions of recombinant heteropolymer ferritins were expressed with different ratios of H to L subunits. The average expression ratios of H:L- and L:H-ferritins were compared by densitometry. H:L- and L:H-ferritins were about 1:2.5 and 3:1, respectively, in both coexpression systems (data not shown). The expression level of H-ferritin was less than that of L-ferritin. These results agree with previous reports, with both prokaryotic and eukaryotic expression systems, where recombinant H-ferritin exhibited low expression in whole-cell proteins [6, 9]. Other reports suggest that H-ferritin might associate with mRNA and inhibit translation of a certain mRNA, which has been demonstrated to suppress cell growth [1, 4]. Additionally, in the present study, the cell growth of the H-ferritin transformant was slower than the L-ferritin transformant with IPTG as inducer. The L-ferritin transformant was shown to be about 1.6-folds higher than H-ferritin at 600 nm after IPTG induction. The above results indicate that H-ferritin may act as a repressor element, which generally affects the expression level of recombinant ferritins and cell growth in *E. coli*.

Heteropolymeric Expression of Recombinant Human Ferritins by Overnight Cultivation

For overcoming suppressed expression of H-ferritin in coexpression, it was reported that H-ferritin was increased by the addition of rifampicin to the media and by the use of an inducible expression system [6]. The addition of rifampicin did not significantly improve the yield of H-ferritin. To solve these problems, the cells were cultured overnight without addition of IPTG; then, expressed samples were observed in SDS-PAGE and Western blotting. In this experiment, for HL-ferritin, the ratio of H- and L-ferritins demonstrated an almost even subunit composition in both coexpression systems (Fig. 2A). The average composition ratios of HL- and LH-ferritins were approximately 1:1.2 and 1.4:1, respectively. The expression levels of HL- and LH-ferritins were about 34%. It is assumed that the results from this experiment are due to cAMP in the BL21 (DE3) strain, which contains the T7 RNA polymerase gene downstream of the *lac*UV5 promoter in genomic DNA. It was reported that if this host was grown in a glucose-limiting medium (LB-media) until the stationary phase, as glucose was consumed, cAMP accumulated inside the cells where it binds to CRP. T7 RNA polymerase was expressed by the cAMP-CRP complex, which binds to the CRP binding region located near the *lac* promoter and enhances transcription [7, 14]. Therefore, our conclusion is that recombinant H-ferritin was expressed, without a suppressive effect as occurred during the log phase using IPTG; however, this was only after cells had reached the stationary phase and consumed all the glucose.

Functional and Morphological Properties of Recombinant Human Ferritins

For identification of heteropolymer formation of coexpressed ferritins, native-PAGE was performed after purification. The yield, purity, and total quantity of the ferritin after each step of the purification procedure are summarized in Table 1. All of the expressed ferritins were found in fully assembled multisubunit complexes on native-PAGE. The electrophoretic migration of the standard H-ferritin homopolymer was faster than that of the L-ferritin homopolymer, resulting in different protein surface charges; whereas the migration of recombinant HL- and LH-ferritin heteropolymers were observed between H- and L-ferritin homopolymer bands because the heteropolymeric ferritin contained complex ratios of H and L subunits (Fig. 3A). To determine whether the assembled ferritins were functionally able to store iron, iron staining was

Table 1. Purification of the recombinant H-, L-, HL-, and LH-ferritin from *E. coli*.

Purification step	Total protein (mg/l)[a]				Yield (%)[b]				Purity (%)[c]				Purification (fold)			
	H	L	HL	LH	H	L	HL	LH	H	L	HL	LH	H	L	HL	LH
Crude extract	725	650	637	1,150	100	100	100	100	26	36	35	34	1	1	1	1
Heat treatment	250	216	210	475	97	65	71	89	73	70	74	73	2.8	1.9	2.1	2.2
$(NH_4)_2SO_4$	220	172	172	336	85	53	58	63	73	72	74	74	2.8	2.0	2.2	2.2
Mono-Q	140	66	124	188	71	26	54	46	95	93	95	96	3.7	2.6	2.8	2.8
$(NH_4)_2SO_4$	70	30	119	168	36	12	52	41	96	95	96	96	3.7	2.6	2.8	2.8
Superose 12	27	16	53	63	14	7	24	16	99	97	99	98	3.8	2.7	2.9	2.9

[a]Each step shown is soluble proteins from ferritin transformants in 1 liter culture.

[b]The percentage yield of ferritin.

[c]The percentage purity of ferritin in each step.

[bc]The value was calculated by protein quantity and image scanning densitometry.

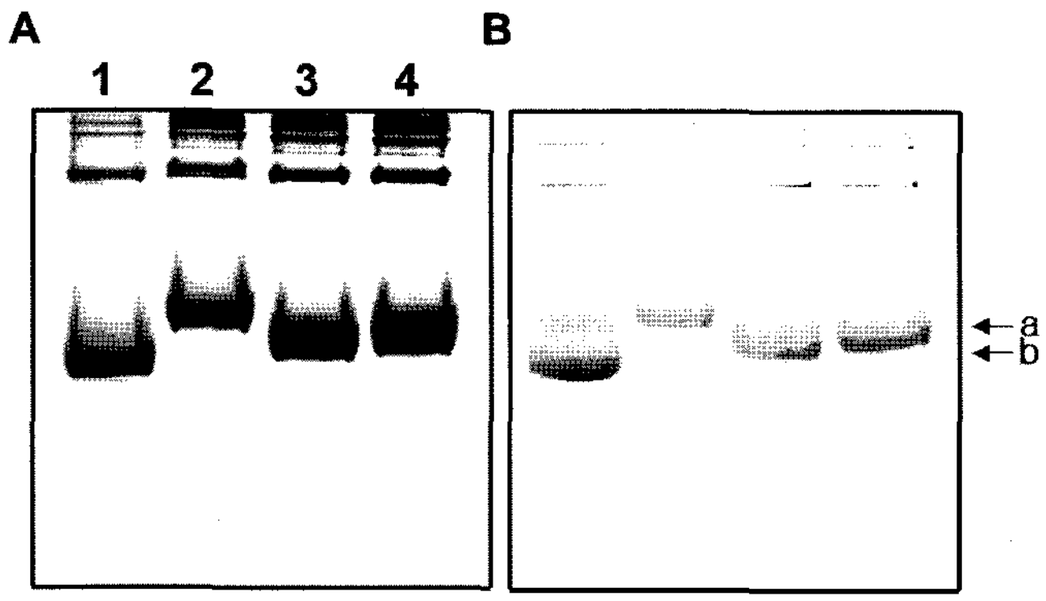

Fig. 3. Native-PAGE and iron staining of recombinant human ferritins.
A. Purified recombinant ferritins were loaded onto a 7.5% Native-PAGE gel and stained with Coomassie blue. **B**. Iron staining was performed after pre-incubation of purified ferritins with ferrous ammonium sulfate. Lane 1, Purified H-ferritin; lane 2, Purified L-ferritin; lane 3, Purified HL-ferritin; lane 4, Purified LH-ferritin. The arrows indicate ferritins (a: LH-ferritin; b: HL-ferritin).

performed in native-PAGE. The recombinant H-, L-, and coexpressed ferritins showed a positive iron band (Fig. 3B). The pattern of electrophoretic mobility was the same as native-PAGE.

Table 2. Molecular mass determination of purified ferritin.

Ferritin type	Ve/Vo[a]	MW (kDa)	Ratio[b]
E. coli H-Ferritin	1.181±0.010	511±16	1
	1.069±0.006	1000±10	0.17
E. coli HL-Ferritin	1.201±0.006	453±10	1
	1.078±0.004	950±7	0.83
E. coli LH-Ferritin	1.207±0.012	432±18	1
	1.079±0.006	940±10	0.67
E. coli L-Ferritin	1.220±0.008	400±13	1
	1.090±0.002	880±3	0.15

[a]Values were obtained from at least three repeated experiments.
[b]Represents the ratio between two peak areas (Area=Au×ml).

Transmission electron microscopy (TEM) was adopted for the study of the morphological properties of recombinant ferritin in *E. coli.* Electron photomicrographs of negatively stained ferritin are shown in Fig. 4. The images of recombinant ferritins showed they were spherical in shape. The particle diameters of recombinant ferritins were measured at about 10 nm, in agreement with that of native ferritin (9–10 nm) *in vivo.* These results provide further evidence for subunit assembly and indicate that recombinant human H- and L-ferritins assemble spontaneously *in vivo* [6, 8] and are functionally similar to native human ferritins [10].

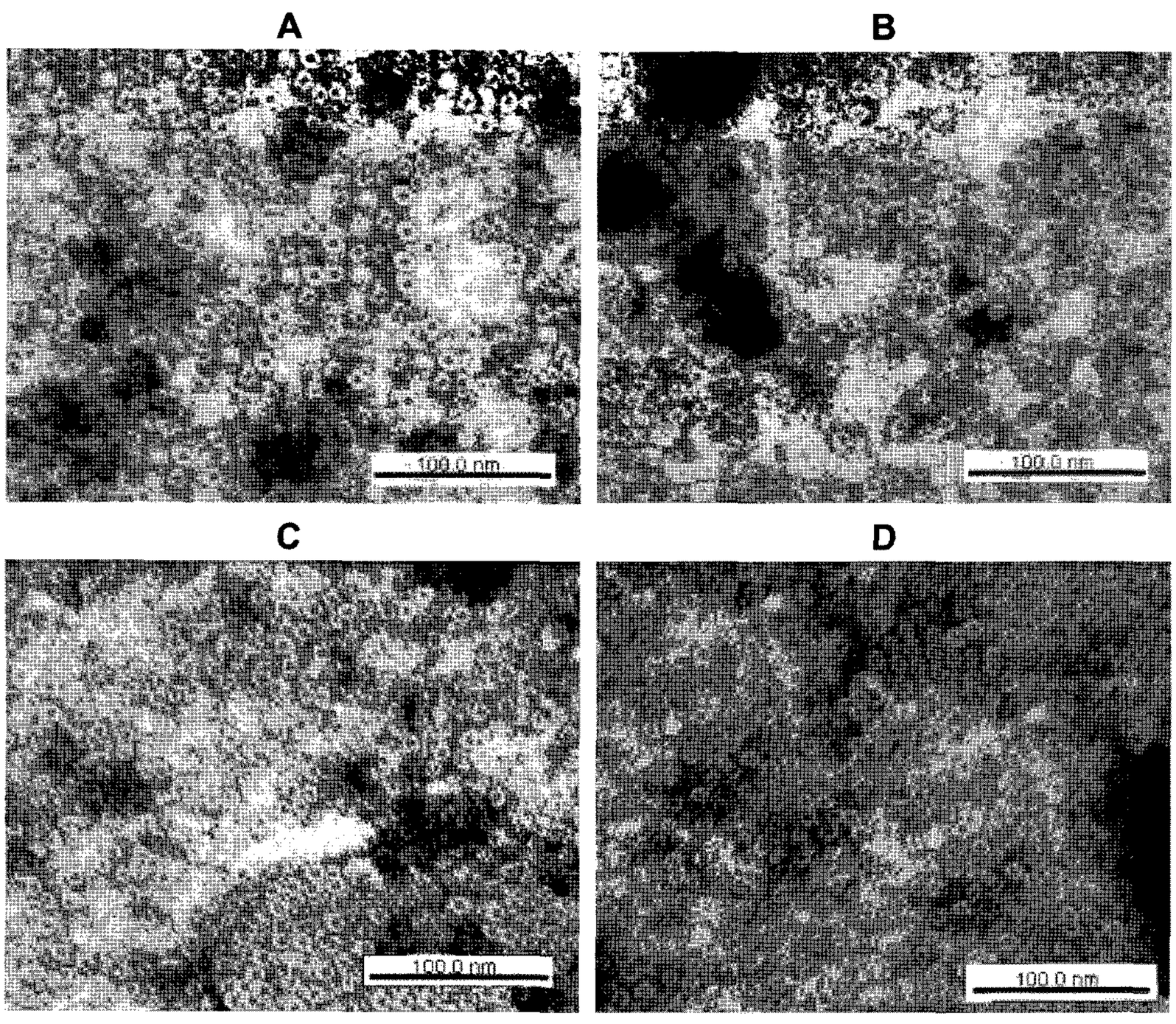

Fig. 4. Transmission electron microscopy of negatively stained purified H-, L-, HL-, and LH-ferritins showing uniform-sized particles.
The core-shell structure is shown in the underfocused images of H-, L-, HL-, and LH-ferritins (**A**, **B**, **C**, and **D**, respectively). The scale bar represents 100 nm.

Molecular Mass Properties of Purified Ferritins from *E. coli*

Four ferritin types were overexpressed in *E. coli* BL21(DE3). The molecular mass of each subunit were estimated to be 21 kDa (H-ferritin) and 19 kDa (L-ferritin) on SDS-PAGE. Moreover, in agreement with previous results, each subunit consisted of multiassembled molecules on native-PAGE and TEM. To determine the molecular mass under native conditions, purified H-, L-, HL-, and LH-ferritins were used. Their molecular masses were calculated from an equation derived from a calibration curve of Ve/Vo against log MW (Table 2). GPC analysis of the purified H- and L-ferritins revealed a major single peak for each with a calculated molecular mass of 511 and 400 kDa, respectively (Figs. 5A and 5B). These results showed that the major peak of recombinant H- and L-ferritins was composed of about 24 subunits. Interestingly, in the case of HL- and LH-ferritins, double peaks were revealed (Figs. 5C and 5D). The molecular masses of HL-ferritins were about 950 and 453 kDa, whereas LH-ferritins were about 940 and 432 kDa. In addition, both H- and L-ferritins revealed a minor peak with a molecular mass of 1,000 and 880 kDa, respectively, before showing a major peak (Fig. 5). These results suggest that part of the expressed ferritin could be a dimer that consists of two intact ferritin multimers. The subunits of ferritin were analyzed by capillary electrophoresis [31], which has not been previously reported regarding two intact ferritin multimers. Heteropolymeric ferritin, especially, showed two major peaks. The first peaks of the heteropolymeric ferritins overwhelmed those of the homopolymeric ferritins with respect to the ratio of peak areas. This heteropolymeric ferritin may suggest that ionic or hydrophobic interaction resulted in weak intermolecular binding. These novel properties of heteropolymeric ferritin are deserving of further study.

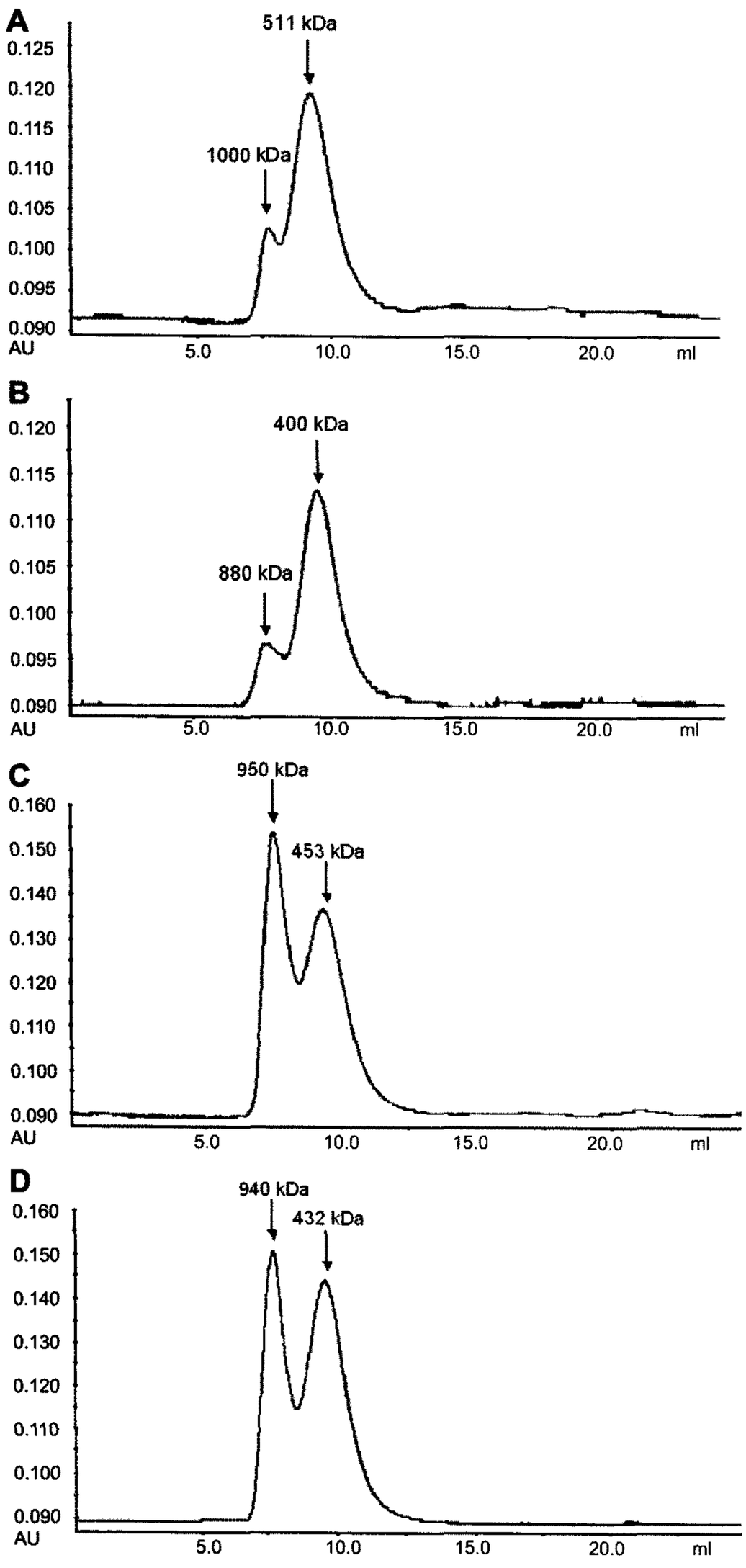

Fig. 5. The elution peaks of H-, L-, HL-, and LH-ferritins (**A**, **B**, **C**, and **D**, respectively) through gel filtration chromatography. The arrows indicate ferritin peaks.

Taken together, recombinant H-, L-, HL-, and LH-ferritins in *E. coli* have a morphological and multiassembly properties *in vivo*. The mechanism of ferritin assembly still remains to be determined by further detailed research. Coexpression vectors may contribute to the further clarification of ferritin assembly and coexpression.

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