J. Microbiol. Biotechnol. (2007), **17**(10), 1695–1699

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Functional Assembly of Recombinant Human Ferritin Subunits in *Pichia pastoris*

LEE, JUNG-LIM[1], CHEON-SEOK PARK[2], AND HAE-YEONG KIM[2]*

[1]*Department of Food Science, Massachusetts Agricultural Experiment Station, University of Massachusetts, Amherst, Massachusetts 01003, U.S.A.*
[2]*Institute of Life Sciences & Resources and Graduate School of Biotechnology, Kyung Hee University, Yongin 446-701, Korea*

Received: June 14, 2007 Accepted: July 24, 2007

Abstract Ferritin is an iron storage protein found in most living organisms as a natural assembled macromolecule. For studying the functional ability of the ferritin assembly, human H- and L-ferritins were expressed and purified from *Pichia pastoris* strain GS115. The recombinant H- and L-ferritins showed a globular form with transmission electron microscopy. The rate of iron uptake for H-ferritin was significantly faster than that for the L-ferritin *in vitro*. By gel permeation chromatography analysis, recombinant ferritins were confirmed as multimeric subunits with high molecular weight and it was indicated that assembled subunits were able to store iron *in vivo*.

Keywords: Human ferritin, multi-assembly, iron, *Pichia pastoris*

Ferritin is an iron storage protein, which is found in mammals, plants, and bacterial cells. It plays an important role in protecting the cell from the damage due to iron-catalyzed production of reactive oxygen species and in keeping iron in a metabolically accessible form [2, 7, 17, 21]. In humans, two types of subunits, H (heavy or heart)- and L (light or liver)-ferritins, have over 50% amino acid sequence homology and these molecules are found in varying ratios in ferritin from different tissues. In general, it was reported that the proportion of each subunit varied from predominantly H-ferritin in heart to predominantly L-ferritin in liver and spleen. Two main subunit types are found in natural assembled molecules *in vivo*. The large sphere constituting a ferritin particle consists of an outer protein coat composed of multimeric subunits and an inner core composed of ferric ions in the form of a crystalline hydroxyphosphate mineral [10–12, 18].

*Corresponding author
Phone: 82-31-201-2660; Fax: 82-31-204-8116;
E-mail: hykim@khu.ac.kr

The molecular characterization of recombinant ferritins has been reported in an *E. coli* expression system [15]. However, the characterization of assembled recombinant human ferritin in *Pichia pastoris* has not been studied for its functional industrial use. Among yeast expression systems, *Pichia pastoris*, in particular, uses the powerful AOX1 promoter to produce higher levels of recombinant protein than *Saccharomyces cerevisiae*. Unlike *S. cerevisiae*, the foreign gene is integrated into the genomic DNA of *Pichia pastoris* [3, 14]. This integration provides genetic stability with *P. pastoris* as the host system, and furthermore, the glycosylated protein structures are more similar to those of humans than hyperglycosylated protein structures in *S. cerevisiae* [4]. In our previous reports, human H-ferritin [8] and L-ferritin [9] were expressed in *Pichia pastoris*. This report describes for the first time the characterization of the ferritin assembly in *Pichia pastoris*. In this study, two kinds of H- and L-ferritin cDNAs were PCR amplified. For production of multi-assembled H- and L-ferritins, both of the H- and L-ferritin cDNAs were PCR amplified and constructed with a *Pichia* expression vector. The purpose of this study was to determine if recombinant human ferritins result in multimeric assembly for functional iron storage as in the native human ferritin *in vivo*.

Materials and Methods

Yeast Strains and Plasmid

Pichia pastoris strain GS115 and expression vector pPICZαA were obtained from Invitrogen (San Diego, CA, U.S.A.). The pPICZαA plasmid contains a zeocin select marker, an alcohol oxidase 1 (AOX1) promoter-terminator cassette, and a multicloning site. It also contains the *EcoI* gene, which enables replication of *E. coli* transformants.

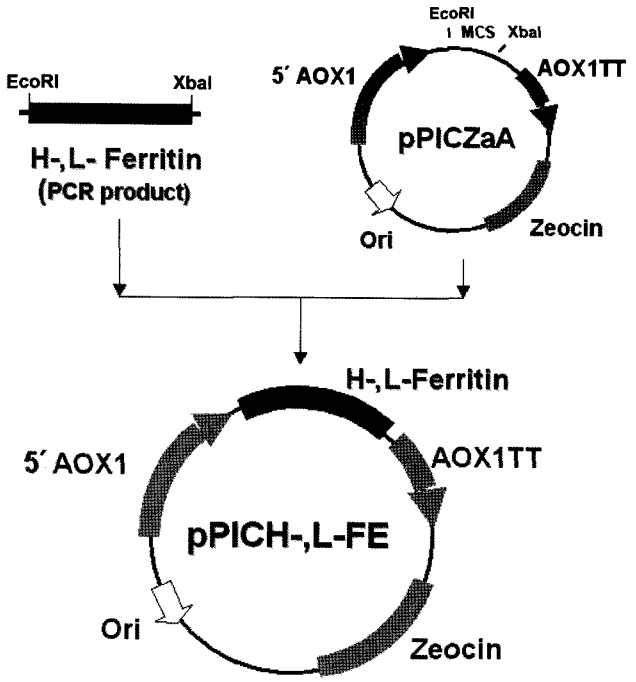

Fig. 1. Construction scheme for expression vectors containing human H- and L-ferritin genes.
The amplified human H- and L-ferritin cDNA regions were inserted into vectors that contained unique EcoRI and Xbal restriction enzyme sites.

Cloning and Transformation

Human H- and L-ferritin cDNAs were amplified by PCR using a heart and liver cDNA library (Clontech, Palo Alto, CA, U.S.A.). The primers for cloning were based on the human H- and L-ferritin sequences from GenBank (Accession #M97164, #M11147) and were commercially synthesized (Bioneer, Daejeon, Korea). The primer sequences were as follows: PichH-Fe5: 5'-GCGCGAATTCATGACGACCG-CGTCCACCTCG-3'; PichH-Fe3: 5'-GCGCTCTAGATT-AGCTTTCATTATCACTGTC-3'; PichL-Fe5: 5'-GCGCGAA TTCATGAGCTCCCAGATTCCTCAGAATTATTCC-3'; and PichL-Fe3: 5'-GCGCTCT AGATTAGTCGTGCTTG-AGAGTGAGCCTTTCGAA-3'. 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.). The PCR product was cloned in-frame into the pPICZaA vector at the 5' EcoRI and 3' Xbal sites (Fig. 1). The constructed vector was linearized with PmeI and transformed into GS115 by electroporation using a gene pulser unit (Bio-Rad, Hercules, CA, U.S.A.) at 1,500 V, 25F, and 200 Ω, and using a 0.2-cm cuvette. Transformants were plated out on YPDS (yeast extract, peptone, dextrose, and sorbitol) plates and incubated at 30°C for 3 days. Several integrated colonies were selected by genomic PCR. The PCR was carried out under the same conditions as described above.

Small-scale Production of Recombinant Human Ferritins in *Pichia pastoris*

Transformants were incubated overnight in buffered minimal glycerol medium (20 ml), harvested, and the cell pellet was resuspended in buffered minimal methanol medium. Cells were cultured in baffled flasks shaken at 250 rpm for 5 days at 30°C. Methanol was added to maintain a final concentration of 0.5% (v/v) methanol. Cells were collected at day 5 to determine expression of recombinant ferritins.

SDS-PAGE and Western Blots

For molecular weight determination, cell extracts were run on a 13% (w/v) SDS-PAGE gel and stained with Coomassie brilliant blue. For detection of ferritin, proteins were blotted onto a nitrocellulose membrane (Schleicher & Schuell, Keen, NH, U.S.A.). The nitrocellulose membrane was incubated with blocking solution containing 1% (w/v) nonfat milk for 1 h and washed with TBST [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] buffer. The H- and L-ferritin specific monoclonal antibody was added as a primary antibody. Consequently, anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody. An ECL kit was used to detect the signal specific for recombinant H- and L-ferritins (Amersham Pharmacia, Uppsala, Sweden).

Purification of Recombinant Human Ferritins

To purify the soluble H- and L-ferritins, cells were extracted using glass beads (Sigma, St. Louis, MO, U.S.A.) and the supernatant was collected by centrifugation. The cell extract was heated at 72°C for 10 min and the supernatant was precipitated with 80% (w/v) saturated ammonium sulfate. After dialysis in 20 mM Tris-HCl (pH 7.4) and 50 mM NaCl, the sample was loaded onto a Mono-Q column. With the use of an FPLC system (Pharmacia, Uppsala, Sweden), the Mono-Q column was eluted with a 0–500 mM NaCl gradient in 20 mM Tris-HCl (pH 7.4). The fraction containing ferritin was collected and concentrated with a microcon 10 membrane (Millipore, Billerica, MA, U.S.A.). The protein concentration was determined using a protein assay kit (Biorad, Hercules, CA, U.S.A.) [13]. The kinetics of iron uptake was determined using 0.1 μM of purified ferritin and BSA. A freshly prepared 100 mM solution of ferrous ammonium sulfate [19] was added to the purified H- and L-ferritins to a final concentration of 0.1 mM at 30°C. The absorbance of the reaction mixture was monitored at 310 nm using a spectrophotometer (Shimadzu, Kyoto, Japan).

Electron Microscopy

Samples of purified recombinant ferritins were prepared for transmission electron microscopy (TEM) by air-drying droplets onto carbon-coated Formvar-covered 200 mesh Cu TEM grids. 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

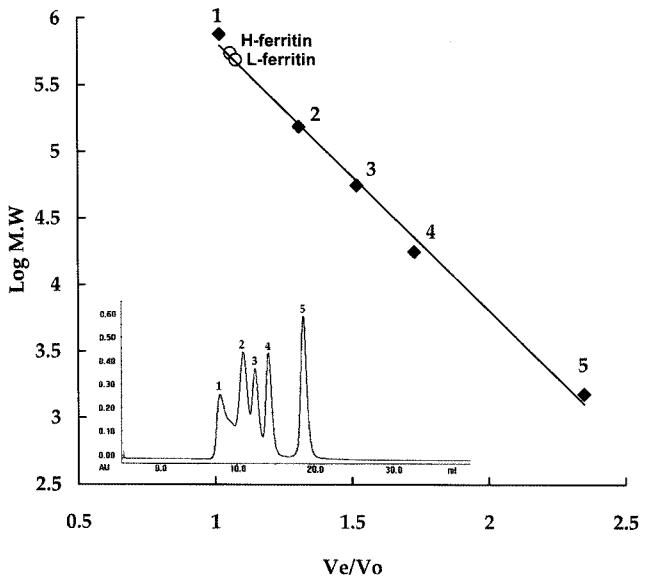

Fig. 2. Calibration plots of standard protein through gel filtration chromatography (GPC) including molecular weight for human H- and L-ferritin.
Relationship between the relative Ve/Vo of GPC and the log of molecular weight from standard proteins (#1-5: varying molecular masses of proteins: 670, 158, 44, 17, and 1.35 kDa, respectively). Inset: The calibration peaks of standard protein through GPC. Plotted values are the means and standard deviations derived from three independent assays.

operating at 100 kV. The length of the calibrated camera was 80 cm. The particle size of ferritin was determined with a bar scale from enlarged photomicrographs [20].

Gel Permeation Chromatography

For calculation of molecular mass, purified H- and L-ferritins were used. Molecular masses of ferritin were determined *via* gel filtration on a Superose 12 column (Pharmacia, Uppsala, Sweden) using a modification of the GPC method of Andrews [1]. Standard protein molecular mass markers were a 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-Cl (pH 7.4) buffer at a flow rate of 0.5 ml/min at room temperature. The elution and void volumes were estimated by an automated FPLC program. Ve/Vo was plotted against the log M.W. The calibration plots of standard protein are shown in Fig. 2.

Results and Discussion

Expression and Purification of Recombinant Human H- and L-Ferritins in *Pichia pastoris*

For expression of human H- and L-ferritins, the transformants were cultured and induced in *Pichia* (GS115 strain) by addition of methanol (0.5% final concentration) for 5 days. The cell extract samples were resolved in SDS-PAGE and the expression of identified ferritin was determined using monoclonal ferritin antibody. Ferritin signal bands were observed in Western blots. The purification of ferritin was performed at room temperature because the ferritin is a thermostable protein [6, 16]. Soluble protein was heat-treated for eliminating cellular protein from transformants. The sample was loaded onto a Mono-Q column of an FPLC system. The fractions containing H- and L-ferritins were collected and concentrated with a microcon 10 membrane. The purified H-ferritin was resolved in SDS-PAGE and determined by Western blotting analysis (Fig. 3). The result showed 3 bands with estimated

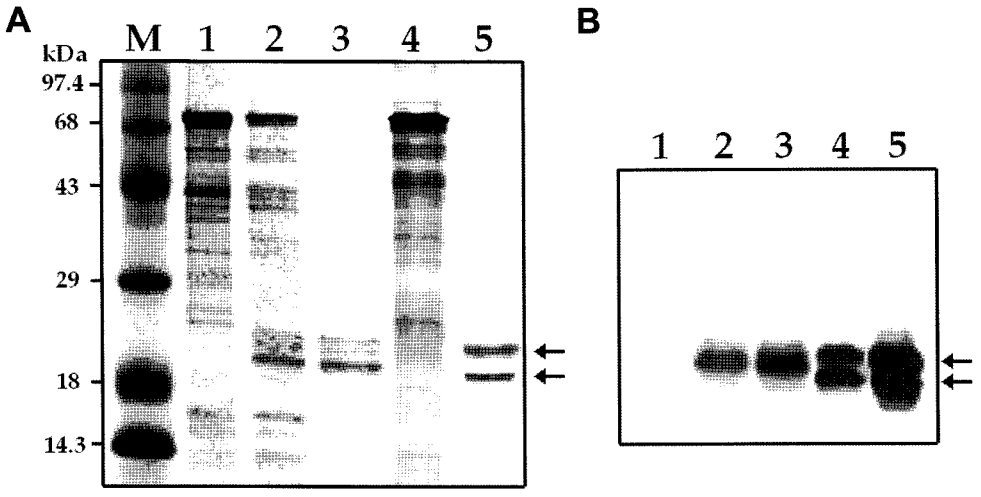

Fig. 3. Expression and purification of recombinant H- and L-ferritins. Samples were loaded onto a 13% SDS-PAGE.
A. Proteins were stained with Coomassie brilliant blue. **B.** Western blot analysis. M: protein standard maker; lane 1: cell extracts from control strain; lanes 2 and 4: cell extracts from transformant; lanes 3 and 5: purified H- and L-ferritins. The arrows indicate ferritins.

molecular mass of 21–22 kDa. The purified L-ferritin yielded 2 bands with estimated molecular mass of about 19–21.5 kDa. This result differed with the reported molecular masses for human ferritin subunits in *E. coli*. In the previous report, expressed human H- and L-ferritins were about 21 and 19 kDa in *E. coli*, respectively [10, 11]. These heterogeneous signal bands were due to differences in posttranslational modification. In the case of ferritin expression, we previously proved that the recombinant human H- and L-ferritins are glycosylated in *Pichia pastoris* [8, 9].

Morphological and Iron-Uptake Properties of Expressed Human Ferritins

TEM was adopted for the study of the morphological properties of recombinant ferritins in *Pichia pastoris*. Electron photomicrographs of negatively stained ferritins are shown in Fig. 4. The electron micrographic image of recombinant H- and L-ferritins generally found them to be spherical. The particle diameter of recombinant ferritin measured about 10–11 nm, which agreed with the particle

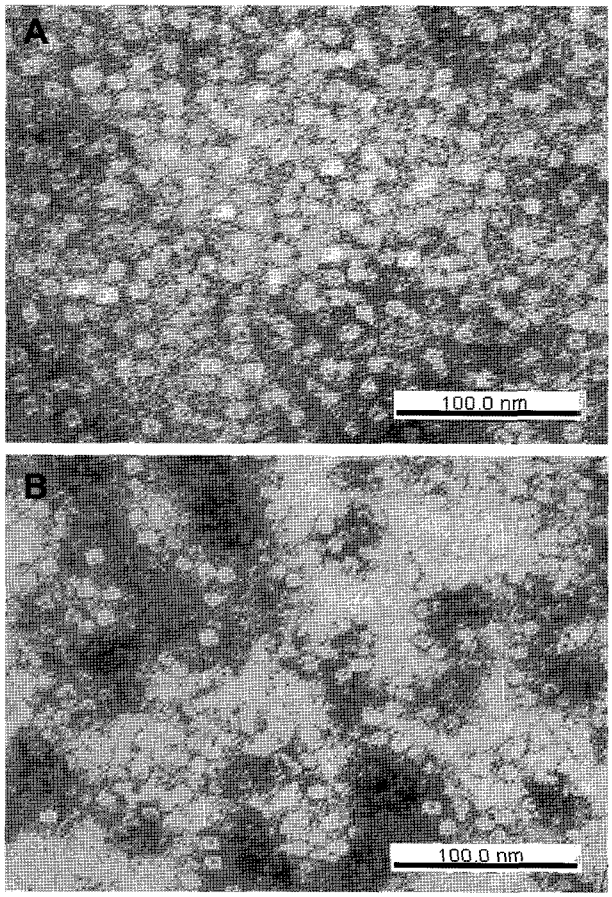

Fig. 4. Transmission electron microscopy of negatively stained purified H- and L-ferritins showing uniform-sized particles.
The core-shell structure is shown in the underfocused images of H- and L-ferritins (**A** and **B**, respectively). The scale bar represents 100 nm.

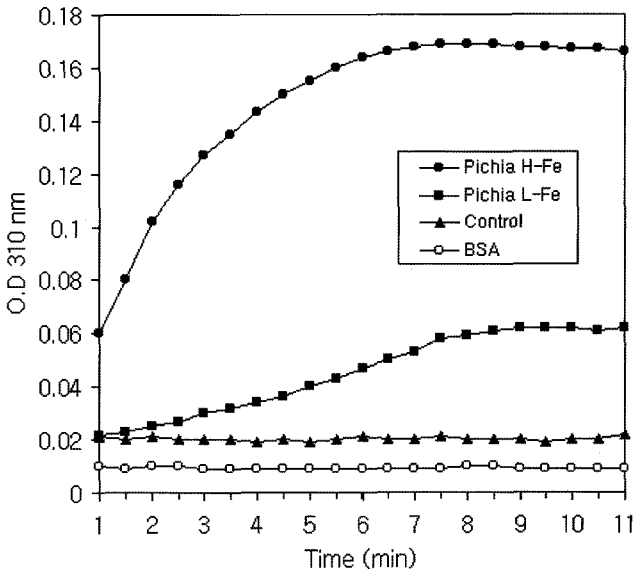

Fig. 5. Iron uptake analysis of purified recombinant H- and L-ferritins.
Ferrous ammonium sulfate (0.1 mM) was added to purified H- and L-ferritins (0.1 mM) and bovine serum albumin (BSA, 0.1 mM) in Hepes buffer (pH 7.0), and the reaction monitored at 310 nm.

size of native ferritin (9–10 nm) *in vivo*. These results provided additional evidence for subunit assembly. They also indicate that recombinant human H- and L-ferritins were assembled spontaneously *in vivo* [4, 6]. Rates of iron uptake were determined by monitoring at 310 nm. Purified H- and L-ferritins and BSA were incubated in the presence of oxygen and a 1,000-fold molar excess of Fe(II) at pH 7.0 [19]. The uptake of iron was much faster in the presence of H-ferritin than in the presence of L-ferritin (Fig. 5). The reaction catalyzed by H-ferritin showed a hyperbolic curve and reached a plateau after 8 min of incubation. The plateau with L-ferritin occurred at about 9 min and was less hyperbolic, whereas the progression plot of BSA was not changed during incubation (Fig. 5). These results are due to the ferroxidase site of human H-ferritin, as previously reported [10–12, 17]. It is suggested that recombinant human H-ferritin has a major role in iron accumulation and oxidation. Recombinant H- and L-ferritins appear to demonstrate a functional property of iron uptake *in vitro*.

Molecular Mass of Purified Ferritin from *Pichia pastoris*

Human H- and L-ferritins were expressed in transformants. The molecular masses of each subunit were estimated to be about 21–22 (H-ferritin) and 19–21.5 kDa (L-ferritin), respectively, on SDS-PAGE and Western blot analysis. Furthermore, as previously noted, each subunit was shown to be a multi-assembled globular molecule on TEM. For determining the molecular mass under native conditions, purified H- and L-ferritins were used. The samples were

loaded onto a gel filtration column of the FPLC system. Their molecular masses were calculated from the equation derived from the calibration curve obtained by plotting Ve/Vo against log M.W. (Fig. 2). GPC analysis of the purified H- and L-ferritins revealed a molecular mass of 596 and 566 kDa, respectively. These results showed that recombinant H- and L-ferritins in *Pichia* each had a higher molecular mass than the recombinant ferritin in *E. coli*, which were 504 kDa and 456 kDa, respectively. It suggests that *Pichia* ferritin was glycosylated in the cytoplasm [4]. The logs of the molecular weights for the ferritins are plotted on a linear standard curve (Fig. 2). The above results showed that recombinant human H- and L-ferritins in *Pichia pastoris* have morphological and functional properties of iron storage *in vivo*. The mechanism of ferritin assembly still remains to be established by further detailed research.

Acknowledgment

The Authors wish to thank Professor Robert E. Levin (University of Massachusetts, Department of Food Science), who reviewed this manuscript.

References

1. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* **91:** 222–233.
2. Arosio, P., T. G. Adelman, and J. W. Drysdale. 1978. On ferritin heterogeneity. *J. Biol. Chem.* **253:** 4451–4458.
3. Bo, X., Y. J. Yang, and Z. X. Huang. 2006. Cloning and overexpression of gene encoding the pullulanase from *Bacillus naganoensis* in *Pichia pastoris*. *J. Microbiol. Biotechnol.* **16:** 1185–1191.
4. Cregg, J. M., T. S. Vedvick, and W. C. Raschke. 1999. Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology (NY)* **11:** 905–910.
5. Grace, J. E. Jr., M. E. Van Eden, and S. D. Aust. 2000. Production of recombinant human apoferritin heteromers. *Arch. Biochem. Biophys.* **384:** 116–122.
6. Guo, J. H., M. Abedi, and S. D. Aust. 1996. Expression and loading of recombinant heavy and light chain homopolymers of rat liver ferritin. *Arch. Biochem. Biophys.* **335:** 197–204.
7. Harrison, P. M. and P. Arosio. 1996. The ferritins: Molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta* **1275:** 161–203.
8. Lee, J. L., H. S. Song, H, J. Kim, J. H. Park, D. K. Chung, C. S. Park, D. Jeoung, and H. Y. Kim. 2003. Functional expression and production of human H-ferritin in *Pichia pastoris*. *Biotechnol. Lett.* **25:** 1019–1023.
9. Lee, J. L., S. N. Yang, C. S. Park, D. Jeoung, and H. Y. Kim. 2004. Purification and its glycosylation pattern of recombinant L-ferritin in *Pichia pastoris*. *J. Microbiol. Biotechnol.* **14:** 68–73.
10. Levi, S., A. Luzzago, G. Cesareni, A. Cozzi, F. Franceschinelli, A. Albertini, and P. Arosio. 1988. Mechanism of ferritin iron uptake: Activity of the H-chain and deletion mapping of the ferro-oxidase site. A study of iron uptake and ferro-oxidase activity of human liver, recombinant H-chain ferritins, and of two H-chain deletion mutants. *J. Biol. Chem.* **263:** 18086–18092.
11. Levi, S., J. Salfeld, F. Franceschinelli, A. Cozzi, M. H. Dorner, and P. Arosio. 1989. Expression and structural and functional properties of human ferritin L-chain from *Escherichia coli*. *Biochemistry* **28:** 5179–5184.
12. Levi, S., P. Santambrogio, B. Corsi, A. Cozzi, and P. Arosio. 1996. Evidence that residues exposed on the three-fold channels have active roles in the mechanism of ferritin iron incorporation. *Biochem. J.* **317:** 467–473.
13. Lieu, H. Y., H. S. Song, S. N. Yang, J. H. Kim, H. J. Kim, Y. D. Park, C. S. Park, and H. Y. Kim. 2006. Identification of proteins affected by iron in *Saccharomyces cerevisiae* using proteome analysis. *J. Microbiol. Biotechnol.* **16:** 946–951.
14. Ro, H. S., M. S. Lee, M. S. Hahm, H. S. Bae, and B. H. Chung. 2005. Production of active carboxypeptidase Y of *Saccharomyces cerevisiae* secreted from methylotrophic yeast *Pichia pastoris*. *J. Microbiol. Biotechnol.* **15:** 202–205.
15. Ro, H. S., H. K. Park, M. G. Kim, and B. H. Chung. 2005. *In vitro* formation of protein nanoparticle using recombinant human ferritin H and L chains produced from *E. coli*. *J. Microbiol. Biotechnol.* **15:** 254–258.
16. Santambrogio, P., A. Cozzi, S. Levi, E. Rovida, F. Magni, A. Albertini, and P. Arosio. 2000. Functional and immunological analysis of recombinant mouse H- and L-ferritins from *Escherichia coli*. *Protein Expr. Purif.* **19:** 212–218.
17. Theil, E. C. 1987. Ferritin: Structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu. Rev. Biochem.* **56:** 289–315.
18. Wade, V. J., S. Levi, P. Arosio, A. Treffry, P. M. Harrison, and S. Mann. 1991. Influence of site-directed modifications on the formation of iron cores in ferritin. *J. Mol. Biol.* **221:** 1443–1452.
19. Waldo, G. S., J. Ling, J. Sanders-Loehr, and E. C. Theil. 1993. Formation of an Fe(III)-tyrosinate complex during biomineralization of H-subunit ferritin. *Science* **259:** 796–798.
20. Wong, K. K., H. Colfen, N. T. Whilton, T. Douglas, and S. Mann. 1999. Synthesis and characterization of hydrophobic ferritin proteins. *J. Inorg. Biochem.* **76:** 187–195.
21. Worwood, M. 1990. Ferritin. *Blood Rev.* **4:** 259–269.