Reconstitution of Iron Cores in Horse Spleen and Yeast-derived Recombinant Human H- and L-chain Ferritins

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Recombinant human ferritin homopolymers (rHF and rLF) were successfully produced in the *Saccharomyces cerevisiae* Y2805, which was transformed with human ferritin H or L-chain genes, respectively. In order to characterize the molecular properties of the recombinant ferritins in relation to mineralization, the proteins were isolated and apoferritins were prepared. The apoferritins were reconstituted with 2000 Fe atoms per protein molecule under various experimental conditions (the concentration of the protein, the buffer concentration of the MOPS buffer, the total volume of the reaction and the reconstitution method). The structure and composition of the iron cores formed in the ferritins were examined using transmission electron microscopy. The recombinant ferritins behaved in a similar manner to other mammalian ferritins in accumulating iron in the core. Proteins of rHF and rLF showed varying reconstitution yields of 37-72% depending on the reaction conditions. In general, the rHF showed higher reconstitution yield than the rLF at the protein concentrations and the reaction volumes we examined. Iron cores with a similar mean particle size were obtained in the rHF, rLF and horse spleen ferritin reconstituted at a protein concentration of 1.0 mg/mL. Electron diffraction of all the three ferritins showed 2-3 diffuse lines, with d-spacings corresponding to those of the mineral ferrihydrite with a limited crystallinity.

Key Words : Yeast-derived recombinant human ferritin homopolymers, Iron core, Reconstitution, Electron microscopy

Introduction

Biomineralization is a process where metal ions are deposited as solid phases in a biological environment *i.e.* living organisms, by absorption, adsorption or by some other mechanism. One of the prominent features on the biomineralization process is that the nucleation and growth of the biomineral phase is carefully controlled by organic matrix biopolymers.[1] An example of a biomineralization within specific compartments or microenvironments is the iron storage protein, ferritin. Protein cages with nanometer-sized cavities provide the reaction environments that control the biomineral size, the specific shape, the chemical composition, the crystal morphology and the crystal structure. Nanoparticles are arising as an important component of advanced materials with its novel optic electrical, magnetic and catalytic characteristics due to their small size.[2,3]

Ferritin, which is a ubiquitous iron protein among living species, is a spherical protein with a diameter of 12 nm and is composed of 24 polypeptide subunits. It contains a 8 nm diameter core, in which a large amount of Fe atoms (up to 4500 atoms) is stored as the mineral ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$). In most vertebrates, ferritins that are present in various tissues consist of two main subunits, heavy (known as heart type, or H) and light (known as liver type, or L). The molecular weights of the subunits are 21 kDa and 19 kDa, respectively.[4,5] Although tissue isoferritins have functional differences that may be related to variations in the subunit composition, the functional differences of the two subunits *in vivo* are not clearly defined. In relation to the *in vitro* function of the subunits, iron cores of *Escherichia coli*-derived recombinant human H- and L-chain ferritins (H- and L-ferritins, respectively) and its variants were previously examined by transmission electron microscopy (TEM). Compared to the H-ferritin, the L-ferritin showed significantly reduced iron uptake rate and cores of increased diameter and regularity upon reconstitution.[6] Electron microscopic studies of ferritin reconstituted with Mn, Al and UO_2 were also performed.[7-10] Such studies provide convenient ways of synthesizing nano-structured minerals with different crystal characteristics in the constrained cavity of the protein. A different approach was also proposed, in which the fabrication of a two-dimensional array of nano-particles was made using ferritin molecules.[11] However, reconstitution studies with recombinant ferritin homopolymers produced in eukaryotic organisms have not been made.

In this study, recombinant human H- and L-chain ferritin homopolymers (rHF and rLF), which are produced in recombinant *Saccharomyces cerevisiae* Y2805, were for the first time subjected to the formation of biominerals, to the best of our knowledge. A variety of reaction factors such as

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the protein concentration, the buffer concentration and the total volume were examined in the reconstitution experiments. Upon reconstitution, the morphology and chemical composition of the nanometer-sized biominerals formed in the recombinant ferritins were analyzed by TEM and electron diffraction.

Materials and Methods

Yeast Strains and Expression of Ferritin from Yeast. *S. cerevisiae* 2805 (*MATa pep4::HIS3 prb1*-d *can1 GAL2 his3 ura3-52*) was used as a host strain for the heterologous expression of the human ferritin H-chain (*hfH*) and L-chain (*hfL*) genes. *S. cerevisiae* cells expressing *hfH* and *hfL* were denoted as strains YGH2 and YGL1, respectively.[12] The horse spleen ferritin was purchased from Sigma. Precultures of the *S. cerevisiae* cells were cultured on a YEP (1% yeast extract) medium supplemented with 2% galactose at 30 °C for 3 days with continuous agitation (200 rpm). The cells were then harvested by centrifugation at 5,000 *g* for 10 min (4 °C) and washed twice with distilled water and once with 20 mM Tris/HCl buffer (pH 7.4). The cells were disrupted in a bead beater (Biospec Products Inc. Okla. USA) for 3 min and the lysates were centrifuged at 10,000 *g* and 4 °C for 10 min.

Purification of the Recombinant Ferritins. The purification of the recombinant H- and L-ferritins from the recombinant yeasts was achieved using a slight modification of the methods reported by Santambrogio *et al.*[13] The rHF was purified by heat denaturation at 70-75 °C for 10 min and 80% ammonium sulfate precipitation followed by gel filtration on Sephacryl S-300. In the case of rLF, a combination of Sephacryl S-200 and DEAE-Sephacel ion exchange chromatography was used to purify the protein from the recombinant *S. cerevisiae*. The purity of the isolated proteins was examined by polyacrylamide gel electrophoresis (PAGE).

Iron Uptake by Recombinant Ferritin. The apoferritins from the purified ferritins were prepared by chemical reduction as described previously.[14] The rate of iron uptake by the ferritin was measured using the method reported by Chang *et al.*[15] Protein concentration was determined by the modified Lowry method.[16] A reaction was initiated by addition of Fe(II) to the apoferritin solution in the Fe/protein ratio of 400. A freshly prepared 2 mM ferrous ammonium sulfate was added to apoferritin (0.5 mg/mL) in 20 mM imidazole buffer (pH 7.0). The iron uptake was observed by measuring the change in the absorbance at 310 nm until the absorbance reached a maximum value (Hitachi U-3300 spectrometer). Additional oxidants were excluded and the Fe^{2+} was oxidized by dissolved oxygen.

Reconstitution of Ferritin. The apoferritin was reconstituted by the addition of a freshly prepared 20 mM ferrous ammonium sulfate in MOPS (3-[N-morpholino] propane sulfonic acid) buffer (pH 7.0). Various reaction conditions including the protein concentration, the buffer concentration of the buffer and the reaction volume was examined. Two different reconstitution methods were performed as previously described by Wade *et al.*[6] One method was performed by the addition of Fe^{2+} in four steps at RT with 1 h intervals to give a calculated loading of 2000 Fe atoms per protein molecule. The other method was carried out by the stepwise addition of Fe^{2+} over 20 times at RT with 15 min intervals to give 2000 Fe atoms per protein molecule. The reaction product was stored at 4 °C for 18 h, followed by extensive dialysis against H_2O. The reconstituted ferritin was filtered using a 0.45 μm filter (Millipore) in order to remove the aggregates that might have occurred during reconstitution. Upon reconstitution, the concentration of iron in the ferritin core was analyzed by a modified Lowry method for proteins[16] and atomic absorption spectrometry (AAS; SpectrAA-400, Varian) for the iron. Unstained samples of the native and reconstituted human ferritins were prepared for electron microscopy by air-drying small drops of the solution onto Formvar-coated copper grids. Samples were negatively stained using 2% phosphotungstic acid to examine the conformation of the protein shells. The electron microscopic images were obtained on a JEOL JEM 2010 operating at 160 keV. Dense areas of the ferritin cores were selected for the electron diffraction patterns. The length of the calibrated camera was 100 cm. The particle sizes were determined by measuring 50 iron cores from enlarged photomicrographs.

Results and Discussion

Reconstitution of the horse spleen and the recombinant human ferritins with Fe^{2+} was traced by an increase in absorbance at 310 nm. The progress curve of Fe^{3+} formation at an iron concentration of 400 Fe atoms per protein molecule among the proteins was compared, as shown in Figure 1. The progress curve of Fe^{2+} oxidation was hyperbolic for rHF, whereas it was sigmoidal for rLF and the horse spleen ferritin (HSF). The reaction of rHF showed the fastest increase in the initial rate and terminated in approximately 5 min, being comparable to that of *E. coli*-derived rHF.[17]

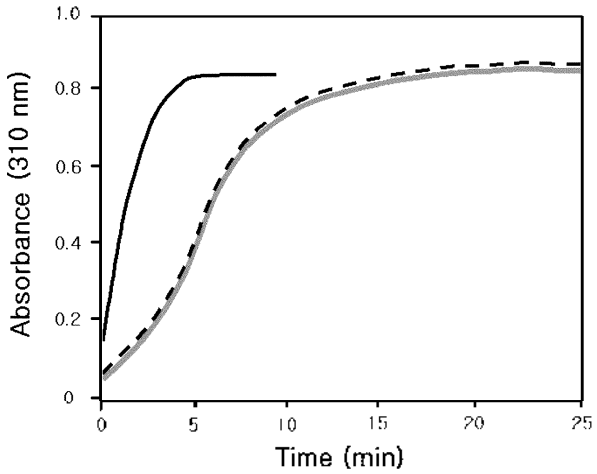

Figure 1. Iron uptake measurement for the human H- and L-chain ferritins, and horse spleen ferritin. The protein concentrations are 0.5 mg/mL and the Fe^{2+}/protein was 400. rHF, solid kinetic curve; rLF, dashed kinetic curve; HSF, gray solid kinetic curve.

Table 1. Reconstitution yield of HSF (horse spleen ferritin) depending on ionic strengths (10 and 20 mM MOPS, pH 7.0), reaction volumes (1.5 and 3.0 mg/mL) and protein concentrations (0.5 and 1.0 mg/mL). The proteins were reconstituted with 2000 Fe atoms per molecule of protein

Theoretical amount[a]	MOPS buffer (mM)	Protein conc. (mg/mL)	Reaction volume	Analytical amount[a,b]	Yield (%)
2000	10	0.5	1.5	1391 ± 57	69.6
	10	0.5	3.0	1449 ± 19	72.5
	10	1.0	1.5	570 ± 12	28.5
	10	1.0	3.0	623 ± 11	31.1
2000	20	0.5	1.5	1221 ± 16	61.0
	20	0.5	3.0	1376 ± 28	68.8
	20	1.0	1.5	1016 ± 66	50.8
	20	1.0	3.0	1201 ± 2	60.0

[a]Values are Fe atoms per protein molecule present in ferritin after reconstitution. [b]Values are obtained from three replicates.

However, the oxidation of Fe^{2+} in rLF terminated in approximately 20 min, appearing faster than the *E. coli*-derived rLF. The result is somewhat comparable to a previous report in that the *E. coli*-derived rHF exhibited a faster uptake rate than *E. coli*-derived rLF.[17,18] The iron uptake of the HSF was similar to that of the rLF under the experimental condition. The result demonstrates that the H- and L-ferritins produced in yeast are as active as the native protein *in vitro*.

The iron concentration and reconstitution yield after reconstituting the HSF with Fe^{2+} ions are shown in Table 1. The iron contents of the HSF (0.5 mg/mL), which were reconstituted with 2000 Fe atoms per protein molecule in 10 mM MOPS (pH 7.0) using either reconstitution method, were analyzed as 1391 ± 57 (reaction volume: 1.5 mL) or 1449 ± 19 (3.0 mL). At a higher protein concentration (1.0 mg/mL), the iron contents decreased to either 570 ± 12 (1.5 mL) or 623 ± 11 (3.0 mL) in the same buffer concentration of buffer solution. A similar tendency was observed: when 1) the reconstitution experiment was performed in 20 mM MOPS (pH 7.0), and 2) iron contents of the HSF (0.5 mg/mL) were analyzed as either 1221 ± 16 (1.5 mL) or 1376 ± 28 (3.0 mL). At a higher protein concentration (1.0 mg/mL), iron concentration decreased to either 1016 ± 66 (1.5 mL) or 1201 ± 2 (3.0 mL) but the decrease was less significant than that observed at the lower protein concentration. When the buffer concentration of the reaction was doubled at high protein concentrations (1.0 mg/mL), the reconstitution yield increased nearly as much as the increase in the buffer concentration. No differences in the reconstitution yield were observed for the different reconstitution methods (data not shown). The reaction volume of the HSF affects the reconstitution yield (Table 1). The reconstitution yield slightly increased as the reaction volume was increased. The maximum yield (72.5%) was obtained when the reaction was performed at 0.5 mg/mL HSF (3.0 mL) in 10 mM MOPS (pH 7.0). On the other hand, the minimum yield (28.5%) was obtained at 1.0 mg/mL HSF (1.5 mL) in 10 mM MOPS (pH 7.0). The reconstitution yield was calculated as the percentage of the iron (μg) incorporated by the proteins per total iron (μg) reacted.

Under electron microscopy, the recombinant ferritins were observed as globular proteins, with sizes comparable to their corresponding tissue ferritins. The reconstituted cores of the rHF and rLF are well identified, which suggests that the recombinant ferritins are well assembled to form a core in a manner analogous to the native tissue ferritins and iron mineralization occurs is in a similar way as in the *E. coli*-derived ferritins.[6] The rHF and rLF were compared after being reconstituted with 2000 Fe atoms per protein molecule in 20 mM MOPS (pH 7.0). The effect of the protein concentration was examined and the protein concentration of 0.5 mg/mL resulted higher reconstitution yields than 1.0 mg/mL (Table 2). Such a tendency is similar to that observed in the HSF. The result indicates that proteins are competitive to the neighboring molecules in accumulating iron atoms as the protein concentration increases in the system, and the

Table 2. Reconstitution yield of ferritin depending on protein concentrations (0.5 and 1.0 mg/mL). The rHF and rLF were reconstituted in two volumes (0.5 and 3.0 mL) of 20 mM MOPS (pH 7.0) with 2000 Fe atoms per molecule of protein

Protein type	Theoretical amount[a]	Protein conc. (mg/mL)	Reaction volume	Analytical Amount[a,b]	Yield (%)
rHF	2000	0.5	1.5	1272 ± 15	63.6
		0.5	3.0	1448 ± 15	72.4
		1.0	1.5	1000 ± 6	50.0
		1.0	3.0	1288 ± 18	64.4
rLF	2000	0.5	1.5	1082 ± 45	54.1
		0.5	3.0	1185 ± 47	59.3
		1.0	1.5	738 ± 32	36.9
		1.0	3.0	1089 ± 17	54.5

[a]Values are Fe atoms per protein molecule present in ferritin after reconstitution. [b]Values are obtained from three replicates.

reconstitution yield is then decreased. Under the experimental conditions, the rHF showed the highest level of reconstitution whereas the rLF showed the lowest. The yield is in the order of rHF > HSF > rLF, which suggests that the reconstitution is related to the content of the H-subunit *i.e.* the ferroxidase activity in this protein. This agrees with the result of the iron uptake kinetics *in vitro* (Figure 1). However, where the iron uptake rate for the HSF (15% H subunit, 85% L subunit) was comparable to the rLF, the HSF showed a higher reconstitution yield than rLF. This result suggests that as the mineralization in HSF progresses, the iron uptake reaction begins slowly in the beginning and the rate increases as the mineral core fills up. Therefore, it indicates that autocatalysis of core formation had more influence on the higher reconstitution yield in HSF. It has been suggested that ferritins composed of more than 18 L subunits among 24 subunits incorporate more iron into the soluble 24-mers than the homopolymers.[19] The reconstitution yield increases as the reaction volume increases (Table 2). When the reaction volume increased by 2 times, the yield increased by 12% on average in the proteins of the rHF, rLF and HSF. The reason for the increase is unclear, but we suspect that the kinetic rate of the Fe ions and the amount of the dissolved O_2 may have influenced on the reconstitution.

Electron photomicrographs of the reconstituted ferritins in the unstained images are shown in Figure 2. The images of the discrete electron dense cores indicate that the reconstitutions are well performed as reported with the *E. coli*-derived ferritins.[6] The reconstituted cores of the rHF and the rLF appear to be generally spherical in shape with the populations being angular (Figure 2a-d). In particular, a large variation in the core size of the rLF was observed. The reconstituted HSF also showed a large population of angular cores with different size variations (Figure 2e and f). The electron dense cores were encapsulated within the protein shells, as shown in the negatively stained images of the ferritins, demonstrating that the minerals are well formed in the cores and some empty cores are left over after mineralization (Figure 2g and h).

Comparisons of the recombinant ferritins reconstituted at the different protein concentrations were made in relation to the morphology and chemical composition of the core mineral. The particle size distribution of the recombinant ferritins and the HSF that was reconstituted under the same experimental condition is compared in Figure 3, and the size data is shown in Table 3. The core size of the rHF (0.5 mg/mL), which was reconstituted with a yield of 72.4%, ranged from 5.89 to 9.70 nm with a mean value of 7.67 nm, and shows a lower size variation. In contrast, the rHF of 1.0 mg/mL that were reconstituted with a yield of 64.4% ranged from 4.33 to 8.03 nm with a mean value of 6.52 nm (Table 3, Figure 3a and b). In the case of the rLF (0.5 mg/mL), the protein reconstituted with a yield of 59.3% shows a core with a mean size of 6.22 nm. The particle size distribution of the rLF was observed over a wide range, as shown on the micrograph. This value increased to 6.92 nm when the rLF (1.0 mg/mL) was reconstituted with a yield of 54.5%. A narrower particle size distribution was obtained in the rLF at this condition (Table 3, Figure 3c and d), but the reason for this is unclear. Previously, Levi *et al.*[18] reported a functional difference between H- and L-ferritins both produced in *E. coli*, and reported that the L-chain ferritin has a higher capacity to induce iron-core nucleation than the H-chain ferritin. In the HSF, the increase in the reconstitution yield from 60.0% (1.0 mg/mL) to 68.8% (0.5 mg/mL) does not

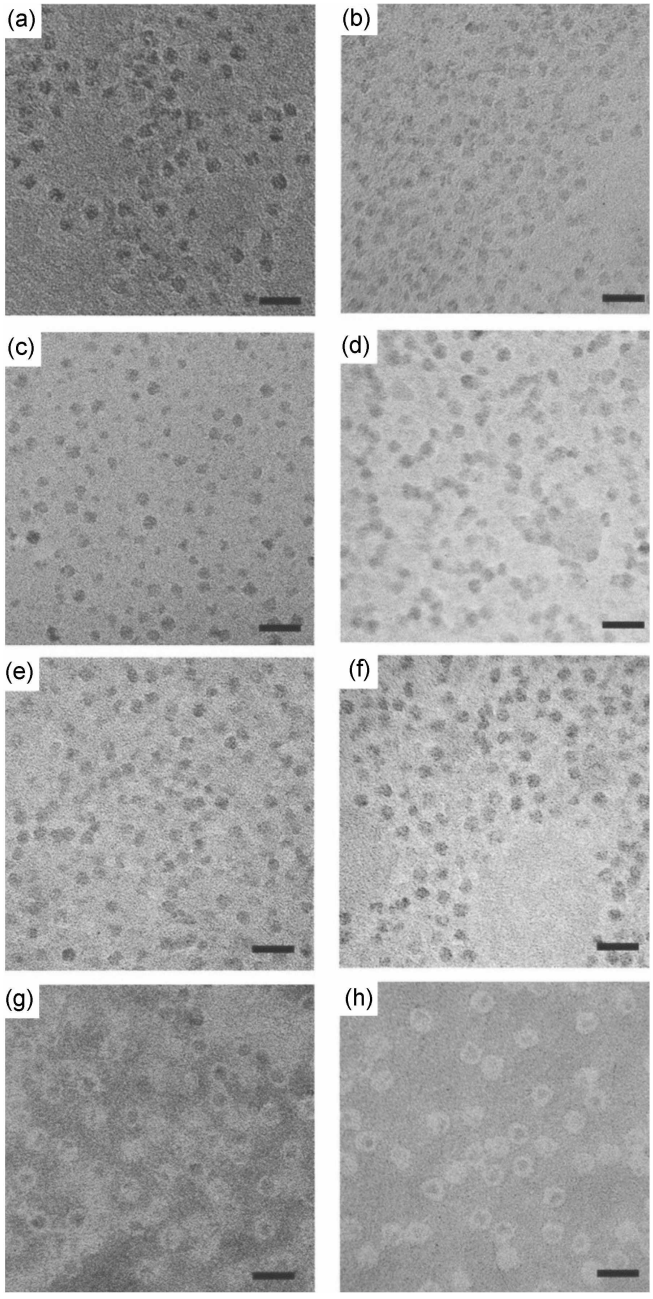

Figure 2. Transmission electron micrographs of the iron cores of the reconstituted rHF, rLF and HSF. The scale bar presents 20 nm in all micrographs. (a) and (b) rHF in two protein concentrations (0.5 and 1.0 mg/mL); (c) and (d) rLF in two protein concentrations (0.5 and 1.0 mg/mL); (e) and (f) HSF in two protein concentrations (0.5 and 1.0 mg/mL); (g) rHF negatively stained in a protein concentration of 0.5 mg/mL; (h) rLF negatively stained in a protein concentration of 0.5 mg/mL.

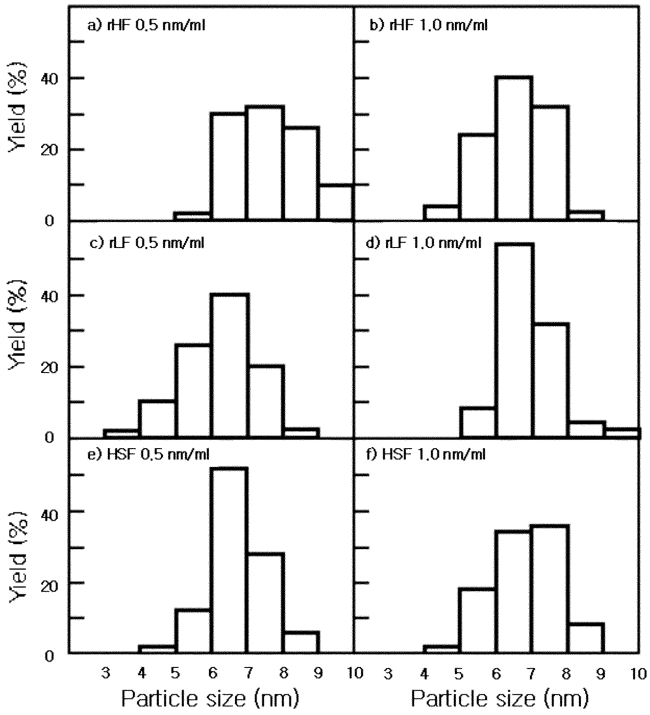

Figure 3. Particle size distributions of the rHF, rLF and HSF.

significantly affect the core size. The core size of the HSF (0.5 mg/mL) ranged from 4.92 to 8.76 nm with a mean value of 6.74 nm whereas the HSF of 1.0 mg/mL range from 4.16 to 8.48 nm with a mean value of 6.67 nm (Table 3, Figure 3e and f). Comparable results were obtained previously where the core size of the HSF (reconstitution yield of 55.0%) 6.17 nm on average.[11] Interestingly, all the ferritins with a protein concentration of 1.0 mg/mL showed somewhat similar core size despite of the variation in the reconstitution yields. However, it was found that there were some differences in the core size of the ferritins of 0.5 mg/mL. The core distribution of the HSF (0.5 mg/mL) revealed a low population of small cores, which also indicates that the reaction is autocatalytic. A comparison of the yeast-derived human rHF with the *E. coli*-derived human rHF showed such a large difference in core size that our yeast-derived rHF was far larger than the *E. coli*-derived rHF.[6] Obviously, the core sizes

Table 3. Particle size data of recombinant human H- and L-chain ferritins, and horse spleen ferritin

Protein type	Protein Conc. (mg/mL)	Particle Mean (nm)	Particle Range (nm)
rHF	0.5	7.67	5.89 - 9.70
	1.0	6.52	4.33 - 8.03
rLF	0.5	6.22	3.91 - 8.76
	1.0	6.92	5.71 - 9.70
HSF	0.5	6.74	4.92 - 8.76
	1.0	6.67	4.16 - 8.48
rHF (s)−	0.5	11.60	9.19 - 13.59
rLF (s)+	1.0	11.30	8.86 - 12.81

−: (s) means stained protein

should be compared carefully since there will be some differences depending on the reaction conditions. There is no report so far on the core size and particle size distribution of ferritin that was reconstituted at the different experimental conditions. It was reported that the core size varies as a result of the reconstitution methods.[6] These results clearly demonstrate that the reconstitution yield is higher with a low protein concentration and a large reaction volume of apoferritin, and the core size of the reconstituted ferritins can vary upon reconstitution.

The differences in the electron diffraction patterns of the proteins were not apparent, because the diffraction lines were diffused and consisted of only three lines at *d* spacings, 0.25 nm, 0.21 nm and 0.12 nm. The minerals were assigned as being a ferrihydrite with them being either amorphous or with a poor crystallinity.

In conclusion, the recombinant ferritin homopolymers that are produced in eukaryotic organisms were first characterized in terms of the molecular properties of the core mineralization. The rHF and rLF behaved in a similar manner to the *E. coli*-derived recombinant ferritins. The effect of the various reaction factors including the protein concentration, the buffer concentration of the MOPS buffer and the total reaction volume on the formation of core minerals was examined. The physicochemical properties of the core minerals were closely related to the proteins and the reaction factors. Therefore, this study demonstrates the potential of synthesizing nano-structured minerals with different crystal characteristics in the core of recombinant ferritin homopolymers obtained from yeast. Further analyses on the magnetic properties of the nano-structured minerals will be of great interest for wide applications.

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