利用昆蟲細胞-桿狀病毒或大腸桿菌表達系統生產大鼠肝臟重鏈儲鐵蛋白同質聚合體所導致其蛋白質結構與功能上的差異

Structural and Functional Differences of Recombinant Rat Liver Heavy Chain Ferritin Homopolymers Produced in Insect Cell-Baculovirus and *Escherichia coli* Expression Systems

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摘要：每個由含 T7 表達載體的大腸桿菌系統 JM109(DE3)所產生之基因重組大鼠肝臟重鏈儲鐵蛋白同質聚合體(rH-Ft-Pol-E)內部已含 158 ± 8 原子的鐵，但由昆蟲桿狀病毒感染昆蟲 *Spodoptera frugiperda* 細胞系統表現的同質聚合體(rH-Ft-Pol-I)則未偵測到鐵原子。兩種來源的蛋白質都是由單一 21 kDa 的次單元體組成；然而，非變性聚丙烯醯胺凝膠的等電點電泳上，rH-Ft-Pol-E 可分離出 3-4 種不同等電點的蛋白質種類，而 rH-Ft-Pol-I 只顯示一種；rH-Ft-Pol-E 的 *p*I 值與羰基含量比 rH-Ft-Pol-I 高。由於大腸桿菌系統在表達同質聚合體時，逕行將鐵原子裝載入儲鐵蛋白質，可能因此造成蛋白質氧化（增加羰基含量），如以圓二色光譜儀檢測，發現 rH-Ft-Pol-E 的二級結構秩序比 rH-Ft-Pol-I 低；當使用血清藍胞漿素系統將鐵原子裝載入儲鐵蛋白質時，兩者也顯示不同的速率與容量。綜括而論，昆蟲細胞-桿狀病毒系統應該比大腸桿菌系統更適合用來生產較符合生理學特性的儲鐵蛋白質聚合體。

Abstract：**Each recombinant H chain rat liver ferritin homopolymers produced in *Escherichia coli* JM109(DE3) using a T7 expression vector (rH-Ft-Pol-E) contained 158 ± 8 atoms of iron, but not one expressed in *Spodoptera frugiperda* cells infected with a recombinant baculovirus (rH-Ft-Pol-I). Both were made up of a 21 kDa subunit; however, the rH-Ft-Pol-E resolved into 3-4 species, whereas the rH-Ft-Pol-I showed only a single species upon a nondenaturing isoelectric focusing PAGE. All of the rH-Ft-Pol-E showed relatively greater *p*Is and carbonyl content than the rH-Ft-Pol-I. Evidence for protein oxidation (increased carbonyl content) from iron incorporation during expression of the homopolymer in the *E. coli* system also resulted in a decrease in ordered secondary structures, compared with the rH-Ft-Pol-I, as analyzed by circular dichroism. Different rates and extent of iron incorporation were also found between the rH-Ft-Pol-E and the rH-Ft-Pol-I using a ceruloplasmin loading system. Taken together, these results suggested that the insect cell-baculovirus system, instead of *E. coli* system, should be used for expression of ferritin H chain homopolymer to obtain physiologically-relevant ferritin.**

關鍵詞：蛋白質表現，儲鐵蛋白同質體，鐵結合，血清藍胞漿素
Keywords: **protein expression, ferritin homopolymer, iron incorporation, ceruloplasmin**

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I. INTRODUCTION

The expression of recombinant rat liver ferritin H chain homopolymer has been successfully performed in insect *Spodoptera frugiperda* (Sf-21) cells infected with a recombinant baculovirus [1]. However, the yield was not great due to a suppressive effect of the expressed ferritin H chain on the growth of the Sf-21 cells [2]. An alternative *Escherichia coli* JM109 expression system, under stringent regulation, was developed for a greater yield of the ferritin H chain homopolymer [2]. Since expression of rat liver ferritin *in vivo* does not require any post-translational modification, the protein folding and structures of the ferritin H chain homopolymers expressed in eukaryotic and prokaryotic cells were thought to be similar. In fact, *E. coli* expression have been frequently utilized for the production of human [3,4] and bullfrog [5] ferritin H and L chain homopolymers. Ferritin homopolymers expressed in the *E. coli* system were mainly used for studying the iron loading functions of ferritin using a Good type buffer system, such as HEPES and MOPES buffers. Some researchers demonstrated that when ferritin was incubated with Fe(II) in HEPES buffer, ferritin accelerated the rate of Fe(II) oxidation and iron incorporation [6,7]. However, we found that iron deposition into ferritin in the presence of Good buffers was directly related to the rate of iron autoxidation which produced damaging oxygen radical species and oxidized amino acid residues in the ferritin [8]. It seems unlikely that such an iron-loading system would occur *in vivo* because no oxidative modification of amino acid residues was found on native ferritin isolated from rat liver [8]. We have demonstrated that it may be more physiological to load ferritin with iron using ceruloplasmin as ferroxidase [8,9].

When the recombinant ferritin H chain homopolymer (rH-Ft) expressed in an *E. coli* system (rH-Ft-E) was purified, we found that it, but not the rH-Ft expressed in an insect cell system (rH-Ft-I), already contained a certain amount of iron. When ceruloplasmin was used to load iron into native rat liver ferritin, rH-Ft-I, and rH-Ft-E, the loading rate and extent of the rH-Ft-E were different from those

of native rat liver ferritin and the rH-Ft-I. We suspected that the structures of the rH-Ft homopolymer had been varied during the expression in the *E. coli* system. The aim of this work was to investigate this aspect of the rH-Ft-E homopolymer. In addition, a possible reason for oxidative damage on the rH-Ft-E homopolymer and the role of ceruloplasmin for loading iron into ferritin are discussed.

II. MATERIALS AND METHODS

1. Materials.

Rat livers and serum were purchased from Pel-Freez Biologicals (Roger, AR, USA). *Spodoptera frugiperda* cells, baculovirus transfer vector pAcUW21, and the linearized baculovirus BaculoGold were purchased from PharMingen (San Diego, CA, USA) and insect cell medium EX-CELL 401 was purchased from JRH Biochemicals (Lenexa, KS, USA). The *E. coli* host JM109 was purchased from Promega Corporation (Madison, WI, USA). The plasmid pBR322 was obtained from Pharmacia Biotech, Inc. (Alameda, CA, USA). The genes for the T7 promoter and the T7 terminator were obtained from plasmid pET-14b, which was purchased from Novagen, Inc. (Madison, WI, USA). The enzymes used for DNA manipulations were purchased from Stratagen Cloning Systems (La Jolla, CA, USA), Boehringer-Mannheim Biochemicals (Indianapolis, IN, USA) or United States Biochemicals (Cleveland, OH, USA) and used according to the manufacturers' instructions. Other enzymes and chemicals were of reagent grade and obtained from Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA).

2. Preparation of native rat liver ferritin and apoferritin.

Ferritin was isolated from rat liver tissue using a method as described as previously [1]. Apoferritin was prepared by using thioglycolic acid and α,α'-dipyridyl, as described by Dognin and Crichton [10]. Protein concentration was determined by the method of Bradford [11] with a Bio-Rad protein assay kit using BSA as a standard. Total iron assays were performed as described by Brumby and Massey [12].

3. Expression of the rH-Ft homopolymer in insect Sf-21 cells.

Manipulation of recombinant baculovirus for expression of rat liver rH-Ft homopolymer in insect cells was described previously [1]. Sf-21 cells were grown at 27°C in serum-free EX-CELL 401 insect cell medium. Cultures of Sf-21 cells at a density of approximate 2×10^6 cells/mL were infected with the recombinant baculovirus containing the rH-Ft gene at a multiplicity of 20 and harvested 4 days postinfection. The cells were harvested by centrifugation at 1,000 *g* and resuspended in 50 mM phosphate buffer (pH 7.0). The cells were subjected to three cycles of freeze-and-thaw and cleared by centrifugation at 100,000 *g* for 30 min. The two-step, nonheated ferritin purification procedure was as follows. The supernatant was applied to a 10-mL DEAE Sepharose Fast Flow column equilibrated in 50 mM Tris-HCl (pH 7.0). The column was eluted using a discontinuous gradient of 50, 100, 150, 200, and 250 mM NaCl in 50 mM Tris-HCl (pH 7.0). The rH-Ft homopolymer eluted around 150-200 mM NaCl. The fractions containing the rH-Ft homopolymer were concentrated and subjected to a Sepharose CL-6B size exclusion chromatography and eluded using 50 mM phosphate buffer (pH 7.0). The fractions containing ferritin were pooled and concentrated with an Amicon concentrator (Centriprep-100, 100-kDa cutoff) (Amicon, Beverly, MA, USA).

4. Expression of the rH-Ft homopolymer in *E. coli*.

The details for the expression of rH-Ft homopolymer were described previously [2]. *E. coli* culture (1 liter) were harvested, centrifuged at 5,000 *g*, and resuspended in 5 mL of 50 mM phosphate buffer (pH 7.0). Cell free extracts were prepared by incubation of the 5-mL cell suspension of *E. coli* with 0.1 g of lysozyme at 37°C for 30 min, followed by three cycles of freeze-and-thaw. The preparation was cleared by centrifugation at 100,000 *g* for 30 min and the rH-Ft-E homopolymer purified by the two-step, nonheated procedure described above. The iron in ferritins was quantitated by protein assay and total iron assay as described previously [2]. Iron content per ferritin molecule was calculated as the atom number of iron divided by the molecule number of ferritin.

5. Gel electrophoresis.

The molecule mass of the subunits of rH-Ft-E and rH-Ft-I were determined by SDS-PAGE using a 12% Ready Gel (Bio-Rad Laboratories, Hercules, CA, USA). The assembly of rH-Ft-E and rH-Ft-I homopolymers were determined by PAGE using a 7.5% Ready Gel (Bio-Rad Laboralories) without SDS. Isoelectric focusing was performed on a Hoeffer 600 series vertical unit using 7.5% nondenaturing polyacrylamide gel (4.5% stacking gel) with a linear gradient, pH 3.5-10.0, according to the instructions of the manufacturer, Pharmacia Biotech, Inc. The isoforms of the rH-Ft-E and rH-Ft-I were visualized by staining of Coomassie blue R (Sigma).

6. Loading iron into ferritins using ceruloplasmin.

The method for loading iron into ferritin using ceruloplasmin has been described previously [1]. Ceruloplasmin was prepared from rat serum as described by Ryan et al. [13]. The iron found in the rH-Ft-E homopolymer was released by using thioglycolic acid and α,α'-dipyridyl as described above to provide apoferritin. Iron loading reactions were conducted by incubation of 0.22 nmol of rat liver ceruloplasmin and 0.22 nmol of various apoferritin in 1 mL of 50 mM NaCl under 37°C. Histidine (550 nmol) chelated Fe(II) (110 nmol) was added and iron oxidation was monitored by optical density at 380 nm. The reactions were terminated by adding 1 mM ferrozine and 1 mM desferrioxamine. The nonincorporated iron was removed by an Econo-Pac 10 DG desalting column. Iron content of ferritin

was analyzed by protein assay and total iron assay as described above.

7. Protein carbonyl content.

The carbonyl content of the various ferritin preparations was determined by the 2,4-dinitrophenylhydrazine method described by Levine et al. [14]. Iron, if any, was removed from ferritin before analysis [10]. The carbonyl content was determined by the absorbance spectrophotometrically at 375 nm using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$ [15].

8. Circular dichroism spectra.

Circular dichroism (CD) spectra were obtained with an Aviv Circular Dichroism Spectrometer (model 62DS) (Lakewood, NJ, USA), using a 0.1-cm path length quartz cuvette. Data was collected at 0.2-nm intervals, and at 10°C, using a temperature-controlled chamber. Spectra were averaged over five scans at a 30 nm/min, and the buffer baselines were subtracted. Protein concentration was 0.15 mg/mL. Only the far UV region from 200 to 260 nm was analyzed due to increased noise at shorter wavelengths.

III. RESULTS

1. Expression of the rH-Ft homopolymers.

The yields of the rH-Ft-E and the rH-Ft-I homopolymers after purification were approximately the same, 1.5 mg/100 mL of cell culture. However, the times required for expression of the rH-Ft-E and the rH-Ft-I homopolymers were significantly different, *i.e.*, 8 h and 4 days, respectively. When the recombinant ferritins were analyzed for total iron and protein, the rH-Ft-E homopolymer contain 158 ± 8 atoms of iron per ferritin molecule, but the rH-Ft-I homopolymer contained no detectable iron. Both the rH-Ft-E and rH-Ft-I homopolymers were soluble and able to be maintained under 4°C for weeks without significant change.

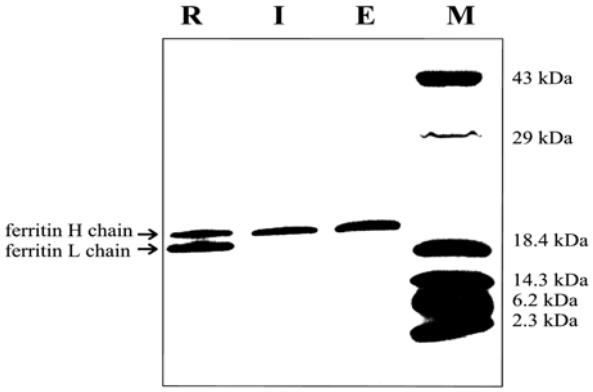

Fig. 1 SDS-PAGE of various ferritins. Approximately 10 μg of the purified rH-Ft-E (lane E), rH-Ft-I (lane I) homopolymers, and native rat liver ferritin heteropolymer (lane R) were subjected to SDS-PAGE on a 12% gel. The *lane M* is molecular mass standards.

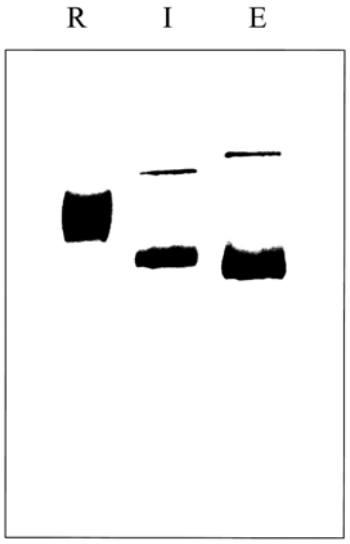

Fig. 2 Nondenaturing PAGE of various ferritins. Approximately 10 μg of the purified rH-Ft-E (lane E), rH-Ft-I (lane I) homopolymers, and native rat liver heteropolymer (lane R) were subjected to PAGE on a 7.5% non-denaturing gel. Both monomers and dimers of various ferritins are shown on the gel.

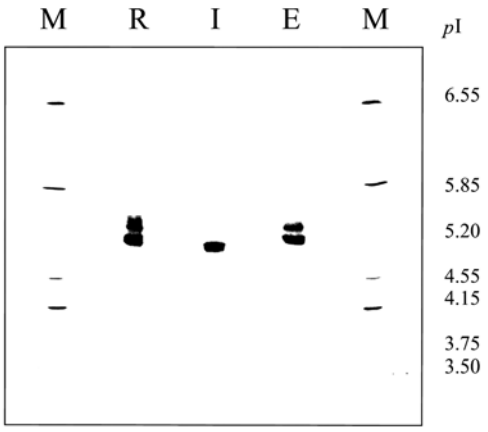

Fig. 3 Isoelectric focusing of various ferritins. The pH range of Ampholine (Pharmacia Biotech) were 3.5-10.0. The protein samples applied to the IEF were 10 μg each lane. The gel was stained with Coomassie blue. Native rat liver ferritin (lane R); the rH-Ft-I homopolymer (lane I); the rH-Ft-E homopolymer (lane E); the isoelectric focusing standards (lane M).

The purified rH-Ft-E and rH-Ft-I were seen as a single band in an SDS-PAGE with an apparent molecular mass of 21.6 kDa, corresponding to the molecular mass of the H chain of native rat liver ferritin (Figure 1). The electrophoretic mobilities of the rH-Ft-E and rH-Ft-I homopolymers on a nondenaturing 7.5% polyacrylamide gel indicated that both the rH-Ft-E and the rH-Ft-I formed multi-subunit complexes (Figure 2). However, the electrophoretic mobility of rH-Ft-E homopolymer was somewhat greater than that of the rH-Ft-I homopolymer. The results suggested that the rH-Ft-E homopolymer might be more

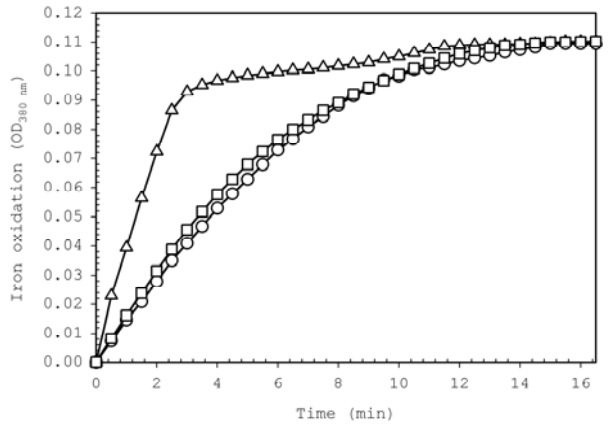

Fig. 4 Iron oxidation during incubation of ceruloplasmin with various ferritins. Ceruloplasmin (0.22 nmol) and apoferritin (0.22 nmol) were incubated at 37°C in 50 mM NaCl, pH 7.0 (1 mL final volume). Histidine:Fe(II) (5:1) was added to a ratio of 500 atoms of iron per ferritin. Iron oxidation was monitored spectrophotometrically at 380 nm. Data represent measurements from at least three individual experiments. Rat liver ferritin (□), rH-Ft-I homopolymer (○), and rH-Ft-E homopolymer (Δ).

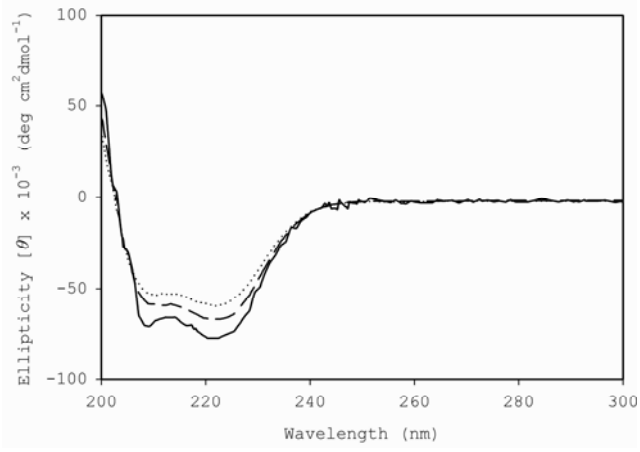

Fig. 5 CD spectra of various ferritins. The CD spectra were obtained in 50 mM NaCl, pH 7.0. The protein concentrations were 0.15 mg/mL and a cell of 0.1-cm pathlength was used for measurements. All the ferritins were prepared as an apo-protein. Solid line is rat liver ferritin. Dashed line is the rH-Ft-I homopolymer. Dotted line is the rH-Ft-E homopolymer.

acidic and/or less molecular mass that the rH-Ft-I homopolymer. Upon isoelectric focusing, a single band was observed with the purified rH-Ft-I homopolymer with an apparent *p*I of 5.19, whereas the rH-Ft-E homopolymer was resolved into several bands with apparent *p*Is between 5.12 and 5.35 (Figure 3).

2. Carbonyl content of ferritin.

The carbonyl contents of native rat liver ferritin, the rH-Ft-I, and the rH-Ft-E homopolymers were 4.5 ± 2.2, 3.5 ± 0.9, and 23.4 ± 2.6 nmol per nmol of protein, respectively.When the ferritins were incubated with 500 molar equivalents of $Fe(NH_4)_2(SO_4)_2$ in 50 mM HEPES buffer (pH 7.0) for 30 min, the carbonyl contents of native rat liver ferritin, the rH-Ft-I, and the rH-Ft-E homopolymers became 53.1 ± 4.4, 85.6 ± 7.3, and 94.0 ± 6.7 nmol per nmol of protein, respectively.

3. Loading iron into ferritins by ceruloplasmin.

Figure 4 shows the repeated oxidation of 110 nmol Fe(II) (chelated with 550 nmol histidine) by ceruloplasmin during incubation with 0.22 nmol of various ferritins in 50 mM NaCl (1 mL). The initial rate and extent of iron oxidation for native rat liver ferritin and the rH-Ft-I homopolymer were similar. When the rH-Ft-E homopolymer was used, iron oxidation was biphasic and the initial rate was approximately 2-fold greater than that for the rH-Ft-I homopolymer and native rat liver ferritin. After iron loading and washing, the rH-Ft-E homopolymer, the rH-Ft-I homopolymer, and native rat liver ferritin all contained approximately 97-98% of the added iron (by total iron assays).

4. Circular dichroism measurements.

As shown in Figure 5, the near UV (240-300 nm) CD spectra of the apo-proteins of the rH-Ft-E, the rH-Ft-I, and native rat liver ferritin were similar. The far UV (200-240 nm) CD spectra of the rH-Ft-E and the rH-Ft-I homopolymers showed two ellipticity minima at about 210 and 222 nm. However, the spectra suggested that the apo-protein of the rH-Ft-I homopolymer had a greater amount of α-helical structure than the rH-Ft-E homopolymer, which suggested that the rH-Ft-E homopolymer contained less ordered secondary structures than the rH-Ft-I homopolymer. The amount of α-helical structure of the apo-protein of the rH-Ft-I and rH-Ft-E homopolymers appeared to be slightly below that of native rat liver ferritin.

IV. DISCUSSION

The rH-Ft homopolymer expressed in the *E. coli* system already contained iron, whereas the homopolymer expressed in the insect cell system did not contain any detectable iron. The question is why the *E. coli* cells, but not the insect cells, provided iron to the expressed rH-Ft homopolymer. It could be possible that some of iron was available in the cytosol of the prokaryotic cells, but not in the insect cells. When the rH-Ft homopolymer was expressed in the *E. coli* cells, this cellular iron may have been oxidized and incorporated. However, *E. coli* cells may not have a ferroxidase-like protein for iron loading into ferritin. Such a protein has been reported in yeast [16] but not in *E. coli*. The mechanism of the oxidation and incorporation of iron into the rH-Ft-E homopolymer in this case might be similar to that using the Good type buffer [6,7]. As we reported previously [8], this kind of iron loading is non-physiological and may oxidize certain amino acid residues to generate carbonyl residues.

The results described here show that the recombinant rat liver ferritin H chain homopolymers expressed in an *E. coli* and an insect cell system consist of a similar molecular mass subunits and both seemed to form multisubunit complexes. However, the rH-Ft-E homopolymer showed greater electrophoretic mobility than the rH-Ft-I ho-

mopolymer on the nondenaturing gel. The rH-Ft-E homopolymer might be modified and contain more basic surface change comparing to the rH-Ft-I homopolymer. the reason might be that some of amino acid residues were modified due to a nonphysiological iron oxidation and incorporation into the rH-Ft-E homopolymer during expression in the *E. coli* cells. In this study, the isoelectric focusing results indicated that the surface charge of the rH-Ft-E homopolymer was indeed more basic than the rH-Ft-I homopolymer. Additionally, we found that the rH-Ft-E homopolymer resolved on the isoelectric focusing gel showed several different *p*Is, whereas the rH-Ft-I homopolymer showed only one. Compared to the unique *p*I value of the rH-Ft-I homopolymer, all the *p*Is of the rH-Ft-E homopolymer were more basic. We have reported that nonphysiological incorporation of iron into ferritin in HEPES buffer may cause the damage of histidine and lysine residue of native rat liver ferritin [17]. This kind of damage would make the rH-Ft-E homopolymer more acidic, instead of basic. However, if we consider that the protein damage may occur as the iron is damaged protein may possibly appear more basic simply deposited into the core of ferritin, the net surface charge of because the more acidic amino acid residues in the protein are not exposed to the surface. We believe that may be the reason why we observed the rH-Ft-E homopolymer showing more basic surface charge than the rH-Ft-I homopolymer on the nondenaturing PAGE and isoelectric focusing gel.

The carbonyl content of the rH-Ft-E homopolymer was 6 times higher than that of native ferritin and the rH-Ft-I homopolymer. Carbonyl formation was also observed upon loading the various ferritins with iron in HEPES buffer. These results suggest that some amino acid residues, especially histidine (which is converted to glutamyl semialdehyde) [13], of the rH-Ft-E homopolymer were oxidatively modified during the nonphysiological iron loading. This kind of modification of rH-Ft-E amino acid residues might also cause the protein conformation changes of the homopolymer and result in changes in its surface charge.

Circular dichroism was also utilized to investigate possible structural differences between the rH-Ft-E and the rH-Ft-I homopolymers. The results suggest that both the rH-Ft-E and the rH-Ft-I homopolymers have a typical CD spectrum and contain a high degree of α-helical structure. However, the circular dichroism analysis indicated that the rH-Ft-E homopolymer contains a less ordered secondary structure, compared with the rH-Ft-I homopolymer. In other words, the secondary structure of the rH-Ft-E homopolymer might partially unfold when some of the amino acid residues were modified. Although the differences of CD spectra between the rH-Ft-E and the rH-Ft-I homopolymers were not great, a minor change might potentially affect the property of the protein. This effect may particularly be seen upon the iron loading into the rH-Ft-E homopolymer using ceruloplasmin as a ferroxidase.

When ceruloplasmin was used to load iron into rH-Ft-E, rH-Ft-I, and native rat liver ferritin, the rH-Ft-I, but not the rH-Ft-E, homopolymer behaved similar to native rat liver ferritin. We reported previously that ceruloplasmin loading iron into ferritin requires an association between protein [18]. This association may enhance the ferroxidase activity of ceruloplasmin. Since the rH-Ft-E homopolymer was modified during expression, its protein conformation might be changed. This alteration might also affect the association of the rH-Ft-E homopolymer and ceruloplasmin and thus result in a biphasic iron oxidation by ceruloplasmin.

The results of the protein structure studies indicate that the protein shell of the ferritin H chain expressed in the *E. coli* system was modified, perhaps due to nonphysiological oxidation of iron in *E. coli*. This protein modification resulted in the ferritin H chain homopolymer having different protein structure, surface charges, and an uncommon iron-loading pattern. The results suggest that the recombinant rat liver ferritin H chain homopolymer expressed in the *E. coli* system, which is quite commonly used, may be not appropriate for iron-loading studies. The results suggest that the expression of the ferritin H chain homopolymer should not be done in an *E. coli* system or more care must be used to avoid damage to the expressed protein. The insect cell-baculovirus expression system may be a better alternative for expression of recombinant ferritin.

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