Refolding of Fusion Ferritin by Gel Filtration Chromatography (GFC)

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Abstract Fusion ferritin (heavy chain ferritin, F_H + light chain ferritin, F_L), an iron-binding protein, was primarily purified from recombinant *Escherichia coli* by two-step sonications with urea [1]. Unfolded ferritin was refolded by gel filtration chromatography (GFC) with refolding enhancer, where 50 mM Na-phosphate (pH 7.4) buffer containing additives such as Tween 20, PEG, and L-arginine was used. Ferritin is a multimeric protein that contains approximately 20 monomeric units for full activity. Fusion ferritin was expressed in the form of inclusion bodies (IBs). The IBs were initially solubilized in 4 M urea denaturant. The refolding process was then performed by decreasing the urea concentration on the GFC column to form protein multimers. The combination of the buffer-exchange effect of GFC and the refolding enhancers in refolding buffer resulted in an efficient route for producing properly folded fusion ferritin.

Keywords: ferritin, refolding, GFC

INTRODUCTION

High-level expression of the cloned gene product as inclusion bodies (IBs) limits the production of genetically engineered proteins from *Escherichia coli*. These aggregates have no known biological activity and it is necessary that the inclusion bodies be solubilized and the protein refolded into its native structure [2]. After the dissolution of IBs, strong chaotropic agents, such as 8 M urea and guanidine hydrochloride, are added to reduce disulfide bonds. Finally, the denatured protein is refolded towards its native conformation in non-denaturation environments. Generally, this is achieved by dilution or dialysis [3]. In recent years, the process of refolding by chromatography methods has been rapidly developed; these methods include ion exchange chromatography, chelating chromatography, hydrophobic interaction chromatography, and gel filtration chromatography (GFC) [4-7] *etc.* Batas [7] indicated that refolding in a GFC packed column was based on an alternative buffer-exchange mechanism to remove high denaturant concentrations.

Ferritin is an iron-binding protein which regulates the iron metabolism or defense mechanism [8]. Natural ferritin has been purified from horse spleen and formulated as a drug for iron supplementation [9]. However, it was banned for the market because of viral contamination. Recombinant technology is, therefore, a good solution for meeting clinical needs in large amounts. Ferritin is expressed as IBs in *E. coli*; the renaturation of the protein is crucial for high production yield [10].

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In the present paper, a method will be described for the refolding and purification of fusion ferritin (heavy chain ferritin, F_H + light chain ferritin F_L) produced in *E. coli* as IBs, in order to obtain the purified ferritin with high activity recovery. Refolding enhancers, such as Tween, PEG, and arginine, were selected and tested for high activity recovery. Ferritin is a multimeric protein with a complex subunit composition from heavy chain (F_H) and light chain (F_L) ferritin monomeric proteins. The high activity of ferritin is associated with a proper formation of shell and core through the combination of F_H and F_L. The mechanism of iron uptake activity is explained as a catalytic role of the protein shell in a complex series of hydrolytic polymerization reactions of Fe (III) [11].

MATERIALS AND METHODS

Chemicals and Primary Separation

Urea, Tween 20, polyethylene glycol (PEG), L-arginine, tris (hydroxymethyl) aminomethane (Tris), and ampicillin were purchased from Sigma. Sephacryl S-200 gel for GFC was obtained from Amersham Biosciences in Sweden. All other agents were of analytical grade.

As the expression host, we used recombinant *E. coli* BL21 carrying fusion ferritin gene, which had been grown in a fermenter containing 1.8 L LB medium with 50 μg/mL ampicillin. Cells were harvested by centrifugation and the collected pellets were re-suspended in lysis buffer and crashed twice by an ultrasonic homogenizer. The cell homogenate was then applied to a centrifuge for IBs collection. The collected inclusion bodies were re-suspended in denaturation buffer (4 M urea and 50 mM

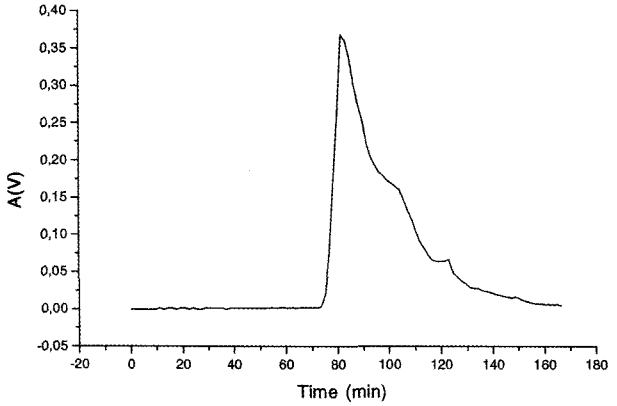

Fig. 1. Refolding chromatogram of ferritin without folding additives in AP-1 column. Ferritin loading: 500 μL at 3 mg/mL. Elution flow rate: 6 mL/cm 2 h. Elution buffer: 50 mM sodium phosphate (pH 7.4). Samples were fractionated every 15 min.

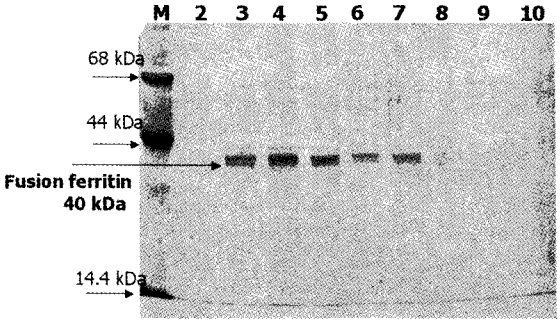

Fig. 2. 15% SDS-PAGE of fractioned samples from Fig. 1.

phosphate buffer at pH 7.4). Details of the fermentation and the preparation of loading sample for GFC are given elsewhere [1].

Gel Filtration Chromatography

GFC in an FPLC system (Pharmacia, Uppsala, Sweden) was carried out at room temperature using an AP1 column (Waters, Milford, MA, USA) packed with 14.5 mL Sephacryl S-200 gel media. The packed column was equilibrated with refolding buffer (50 mM phosphate buffer at pH 7.4 with or without refolding additives, such as Tween, PEG, and L-arginine). 500 μL of denatured protein sample was loaded through a sample loop and eluted with the same buffer at a flow rate of 6 mL/cm^{2}h. The elution fractions were collected and assayed for total protein concentration, ferritin activity, HPLC, and SDS-PAGE analysis.

Assay Methods

The total protein concentration was determined by Coomassie Brilliant Blue assay [12] using bovine serum albumin as a reference. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, according to the method of Laemmli [13]. The concentration of running gel was 15% and that of the stacking gel was 5%. The biological assay was performed by iron storage capacities, which were examined by analyzing the amount of unbound free iron after incubation with ferric chloride [14].

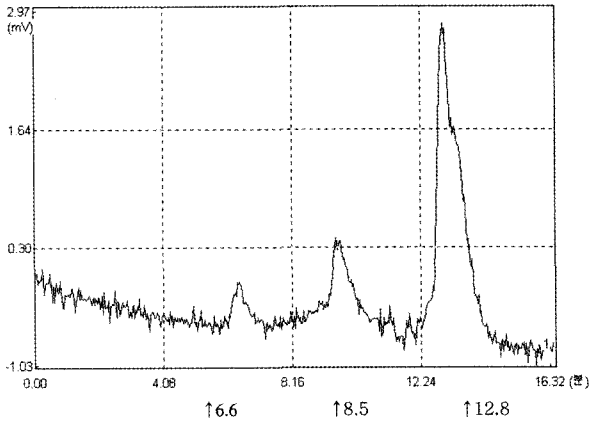

Fig. 3. Chromatogram of GF-HPLC of ferritin without folding additives. Elution flow rate: 0.6 mL/min. Ferritin loading: 20 μL. Wave length: 220 nm. Equilibrium and elution buffer: 50 mM NaH_2PO_4 (pH 6.8). The retention time of native ferritin (450 kDa) is 8.5 min.

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed to discriminate the folded ferritin from contaminants by using a Hypersil C8 column (45 mm ID × 150 mm L). Acetonitrile and deionized water were mixed in a ratio of 25/75 with 0.1% TFA and eluted at a flow rate of 1.0 mL/min. Additionally, a BIOSEP-SEC-S300 (Phenomenex, 45 mm ID × 300 mm L) column was used for gel filtration high performance liquid chromatography (GF-HPLC). The phase was 50 mM NaH_2PO_4 (pH 6.8) with a flow rate at 0.6 mL/min. Detection wavelength was 220 nm.

RESULTS AND DISCUSSION

The use of GFC is a process in which the protein refolding is initiated and the purification of protein from contaminants is simultaneously achieved. The 0.5 mL sample of denatured ferritin in the denaturing buffer is injected into the GFC column equilibrated with the refolding phosphate buffer. Fig. 1 shows the chromatogram of ferritin refolding by GFC where the first main peak refers to ferritin, and the following peaks refer to small molecules. Ferritin fractions 3~7 were detected by SDS-PAGE analysis, as shown in Fig. 2. The monomeric form of fusion ferritin has a molecular weight of 40 kDa, which was calibrated by molecular markers of 14.4, 44, and 68 kDa proteins.

The resolution of the AP-1 GFC column was high enough for molecular weight determination that the pool of fractions 3~5 would be reassayed by GF-HPLC. Fig. 3 shows that the ferritin (450 kDa) which contains ap-

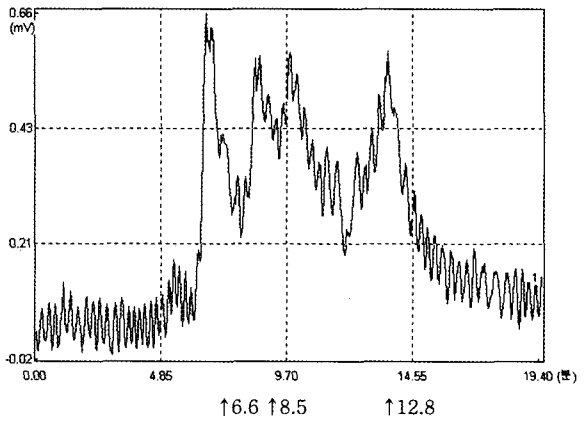

Fig. 4. Chromatogram of GF-HPLC of Tween 20-treated ferritin. Elution flow rate: 0.6 mL/min. Ferritin loading: 20 μL. Wave length: 220 nm. Equilibrium and elution buffer: 50 mM NaH2PO4 (pH 6.8). The retention time of native ferritin (450 kDa) is 8.5 min and the retention time of albumin (44 kDa) is 9.7 min.

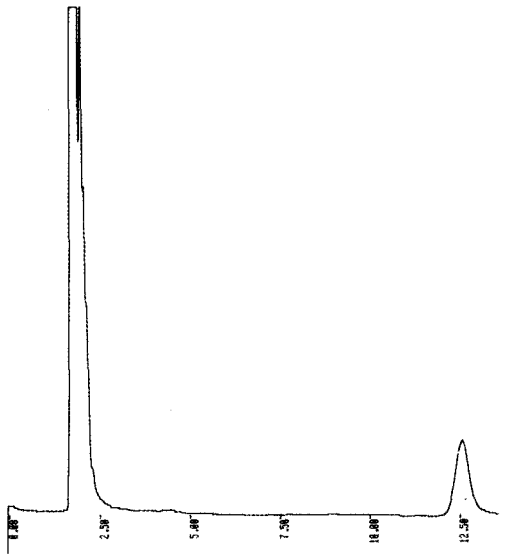

Fig. 5. Chromatogram of RP-HPLC of ferritin without folding additives. Loading: 20 μL. Elution flow rate: 1.0 mL/min. Wave length: 220 nm. Area of first to second peak = 80:8.

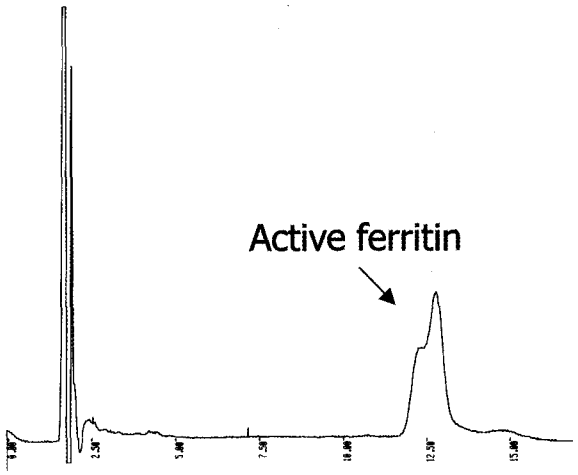

Fig. 6. Chromatogram of RP-HPLC of Tween 20-treated ferritin. Loading: 20 μL. Elution flow rate: 1.0 mL/min. Wave length: 220 nm. Area of first to second peak = 38:36.

proximately ten monomers has a retention time of 8.5 min, and the contaminants correspond to the peak at 12.8 min. As seen in Fig. 4, the peak height of 8.5 min retention time relatively increases by the refolding process with Tween 20 detergent. Tween 20 detergent helps ferritin monomers to form multimeric ferritins. The first two peaks correspond to the multimeric ferritins and the third peak was found to be monomeric ferritin. The peak at 6.6 min seems to be a ferritin which contains more than ten monomers.

Table 1 illustrates an increase in the activities of ferritin treated with folding additives such as Tween 20, PEG, and L-arginine. The additive is a key factor for mediating the refolding of denatured protein [15]. As a detergent, Tween 20 may not only control hydrophobic interactions of ferritin monomer, but also influence the solubility and stability of the native, denatured intermediate states during refolding. The activity of ferritin decreased as the concentration of Tween increased from 0.1% to 0.5% (v/v). Therefore, it is reasonable to postulate that concentrations of Tween 20 in the refolding buffer which are too high could prevent the proper shell and core formation of ferritin monomers. PEG [16] does not behave as a good activity enhancer, as activity increases less than 10%. In contrast, L-arginine is a useful refolding additive, which enhances activity by more than 50%.

RP-HPLC and GF-HPLC were performed to understand the activity change by HPLC [17]. Fig. 5 is the RP-HPLC chromatogram of ferritin without folding enhancer. The peak area ratio between the first and the second peaks equals 80:8. The second peak corresponding to refolded active ferritins increases in size in Figs. 6 and 7. The ratio of the two peaks in Figs. 6 and 7 is 38:8. This observation implies that the folding enhancers of Tween 20 and PEG act as helpers to promote the formation of multimeric ferritins, which are more hydrophobic and

Table 1. Results of iron uptake assay of ferritin with and without folding additives

	No additive	0.1% (v/v) Tween 20	0.1% (v/v) PEG	0.5 M L-arginine	0.2% (v/v) Tween 20	0.5% (v/v) Tween 20
Activity (μM Fe/min)	8.1	10.1	8.7	14.5	6.9	6.6

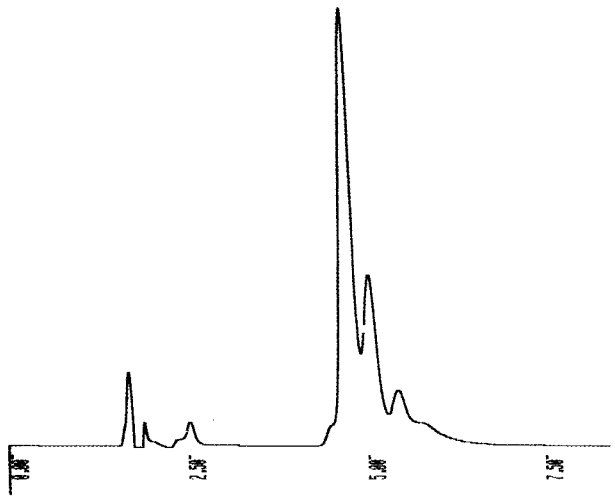

Fig. 7. Chromatogram of RP-HPLC of PEG-treated ferritin. Loading: 20 μL. Elution flow rate: 1.0 mL/min. Wave length: 220 nm. Area of first to second peak = 38:36.

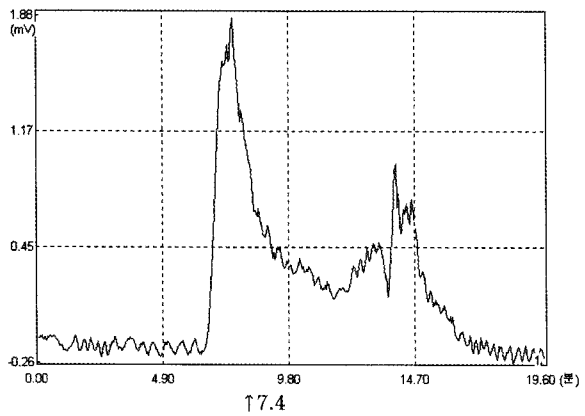

Fig. 8. Chromatogram of GF-HPLC of 0.5 M L-arginine-treated ferritin. Elution flow rate: 0.6 mL/min. Wave length: 220 nm. Equilibrium and elution buffer: 50 mM NaH_2PO_4 (pH 6.8). The retention time of fractioned sample is 7.4 min. The retention time of ferritin is 7.4 min (800 kDa).

have longer retention times than monomeric ferritins. PEG- associated ferritin peaks in Fig. 7 show that there are heterogenous forms of multimeric ferritin revealed by RP-HPLC. This phenomenon could be ascribed to the lower enhancing effect on the activity by PEG. Fig. 8 explains the highest activity of ferritin by L-arginine, ferritin is assembled with the help of L-arginine. Large macromolecules of ferritin at 7.4 min are capable of containing more iron atoms inside the core surrounded by ferritin shells.

CONCLUSION

Using the process based on the GFC technique, the ferritin can be successfully refolded and simultaneously purified from the inactive IBs. To overcome the high insolubility of the protein, a reliable method was developed for recovering recombinant ferritin by the use of 4 M urea in the loading buffer, as well as the refolding additives of Tween 20 and L-arginine. The presence of L-arginine generates an increase of activity of approximately 50%. The recovery of bioactive ferritin from IBs is based on the first dissolution in 4 M urea and the GFC process for ferritin refolding. This is a valuable method that could obtain large amounts of natural and active ferritin for the purposes of clinical therapy.

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