Application of crossflow ultrafiltration for scaling up the purification of a recombinant ferritin

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ABSTRACT

Ferritin proteins are taking center stage as smart nanocarriers for drug delivery due to their hollow cage-like structures and their unique 24-meric assembly. Among all ferritins, the chimeric *Archaeoglobus* ferritin (HumFt) is able assemble/disassemble varying the ionic strength of the medium while recognizing human TfR1 receptor overexpressed in cancer cells. In this paper we present a highly efficient, large scale purification protocol mainly based on crossflow ultrafiltration, starting from fermented bacterial paste. This procedure allows one to obtain about 2 g of purified protein starting from 100 g of fermented bacterial paste. The current procedure can easily remove contaminant proteins as well as DNA molecules in the absence of expensive and time consuming chromatographic steps.

1. Introduction

Ferritins are members of a superfamily of iron storage/detoxification proteins, found in all living systems with the exception of yeasts [1,2]. From a structural point of view, they consist of 24 subunits that assemble to form a spherical nanocage with an external diameter of about 12 nm and an internal diameter of 8 nm. Each monomer consists of a bundle of four antiparallel α-helices (A–D) plus a short fifth helix (E) pointing inside the protein cavity, roughly at 60° to the principal helix bundle. An unfolded loop traverses the full length of the helical bundle and connects the helices B and C [3,4]. Ferritin nanocages are able to self-assemble in a 24-mer protein shell and the dimers are the first intermediate in this pathway [5,6].

Mammalian ferritins are typically made of two types of subunits, namely H (heavy, 21 kDa) and L (light, 19 kDa), whose proportion is different in different tissues. H chain subunit is able to oxidize Fe(II) to Fe(III), while the L one lacks catalytic activity but holds a micro-environment that facilitates iron nucleation and mineralization [7,8]. Ferritin nanocages can load up to 4500 iron atoms in the form of highly insoluble $Fe(OH)_3(H_2O)_3$.

Despite significant differences in the primary sequences, mammalian and bacterial ferritins share similar structures and properties [9]. However, significant differences in quaternary structure and assembly have been described for *Archaeoglobus fulgidus* ferritin (AfFtn) [10]. In fact, whereas ferritins display a "closed" octahedral (4-3-2) architecture, the AfFtn exhibits a tetrahedral (2–3) symmetry resulting in the appearance of four large triangular pores (~45 Å wide) in the protein shell. This "open" quaternary structure is unique among all other known ferritins. Another typical feature of AfFtn is the ability to assemble/disassemble depending on the salt concentration. The protein is fully assembled at high ionic strength (0.5 M NaCl) or in the presence of divalent cations (10 mM $MgCl_2$) [11].

Due to their self-assembly properties, hollow cavity, monodispersity, thermal stability (up to 80 °C), pH stability, biocompatibility, ferritins are taking center stage as smart nanocarriers for drug delivery, especially for the treatment of cancer cells [12]. This kind of cells overexpress TfR1 receptor, that is involved in transferrin uptake, but is also able to bind and internalize circulating ferritin [13,14]. In the last years, in fact, many research groups have exploited the potential of ferritin by loading it with conventional anticancer drugs, contrast agents, therapeutic proteins and/or incorporating various cargoes and targeting moieties on the surface [15–18]. Drug encapsulation can be easily achieved *in vitro* by disassembling the 24-mer by changing the pH, while the reassembly of the shell occurs spontaneously when the pH is restored [19]. However, a significant loss of protein is a typical drawback of this kind of procedure and cargoes

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could be quite unstable upon pH changing. *Archaeglobus* ferritin might overcome these problems as it is able to assemble/disassemble at neutral pH values just changing the ionic strength of the medium [11]. AfFtn has been engineered by grafting H-ferritin BC loop resulting in a chimeric protein (humanized ferritin, HumFt) being able to recognize TfR1 receptor and to be consequently internalized within the cells [20]. Thus, among all known ferritins, HumFt can be considered as a unique tool for targeted delivery of therapeutics and contrast agents. Considering the large number of potential applications of this new chimeric construct, it is of a paramount importance to develop a purification protocol that allows to obtain a large amount of highly purified protein.

In this paper, we develop a highly efficient, large scale, chromatography-free purification protocol mainly based on crossflow ultrafiltration, starting from fermented bacterial paste. Ultrafiltration is one of the most used form of membrane base tangential flow filtration and is used to separate proteins form buffer components, for buffer exchange, desalting or concentration. It is gaining great attention, especially for large scale protein purification, due to its outstanding technical and economic advantages [21,22].

The purification method we set up has the advantage to easily eliminate contaminant proteins as well as DNA molecules, that usually co-purify with ferritins affecting the association/dissociation equilibrium. This aspect is of great importance in view of the therapeutic applications of HumFt as a nanocarrier.

2. Materials and methods

2.1. Bacterial cell growth

The synthetic gene encoding humanized ferritin (humft) optimized for the expression in *E. coli* cells was subcloned into a pET30a plasmid (Novagen) between *Nde*I/*Hind*III restriction sites. A stop codon was added before the *Hind*III recognition sequence. The recombinant expression vector pET30a-humft was transformed into BL21(DE3) competent cells. Protein expression was set up optimizing IPTG concentration, time and temperature of induction. IPTG was tested at three different concentrations (0.1, 0.5 and 1 mM); induction was carried out for 4, 8 and 16 h at 25 and 37 °C. Customized large scale protein production was performed by GeneScript under high density fermentative condition (induction with 0.5 mM IPTG at 25 °C for 16 h).

2.2. Purification strategies

100 g of bacterial paste were resuspended in 1 L of 20 mM HEPES buffer containing 50 mM $MgCl_2$ and four cOmplete™ Protease Inhibitor Cocktail Tablets (Roche). Cells were disrupted by sonication and the soluble fraction was treated with 20% $(NH4)_2SO_4$ for 1 h under stirring at room temperature. The pellet was removed by centrifugation at 15000 rpm for 20 min while the supernatant was extensively dialyzed versus 10 mM sodium phosphate buffer pH 7.2 containing 20 mM $MgCl_2$. 20 mg of deoxyribonuclease I from bovine pancreas (SigmaAldrich) were then added and the reaction was carried out for 1 h at 37 °C. The sample was subjected to a first heat treatment at 62 °C for 10 min and, after centrifugation, to a second one at 72 °C for 15 min. Denatured *E. coli* proteins were removed by centrifugation at 15000 rpm at 4 °C for 30 min. The soluble fraction was further clarified by disposable Sartolab® Vacuum Filters System (Polyethersulfone, 0.22 μm) using 20 g of Sartoclear Dynamics® Lab Filter Aid (Sartorius) containing highly pure diatomaceous earth (Celpure® C300 – pharmaceutical grade) pre-wetted with ultrapure water. DNA removal was performed in a single step by means of crossflow ultrafiltration using a single Vivaflow 200 module (Sartorius) with a cutoff of 100 kDa, coupled to a Masterflex L/S pump system. The same device was also used to exchange the buffer with 20 mM HEPES pH 7.4 containing 50 mM $MgCl_2$. The feed flow rate was set to 40 mL/min (transmembrane pressure: 1.5 bar), both in concentration and diafiltration modes.

Protein purity was monitored by SDS-PAGE, using Mini-PROTEAN® TGX Stain-Free™ Precast Gels (Biorad). DNA removal was followed by measuring 260/280 nm ratio using a Jasco V-650 spectrophotemeter (JASCO Deutschland).

2.3. High performance size-exclusion chromatography (HP-SEC)

HP-SEC was performed using an Agilent Infinity 1260 HPLC apparatus equipped with an UV detector. Separation was carried out using an Agilent AdvanceBio SEC 300 Å, 7.8 × 150 mm, 2.7 μm, LC column connected to an AdvanceBio SEC 300 Å, 7.8 × 50 mm, 2.7 μm, LC guard column. Isocratic analysis was carried out with 20 mM HEPES buffer pH 7.4, 50 mM $MgCl_2$ as mobile phase. Flow rate was 0.7 mL/min over an elution window of 7 min. HumFt elution was followed using the UV detection at 280 nm. Protein standards were prepared in the same solution as the mobile phase.

3. Results and discussion

HumFt nanocarrier has been developed in our lab combining human H ferritin capability of recognizing TfR1 receptor and AfFtn salt mediated association properties. As previously reported [20], HumFt was expressed in *E. coli* cells under the control of an inducible promoter and purified at lab scale level by means of a standard ferritin purification protocol involving ammonium sulfate precipitation and gel filtration chromatography. This procedure allows to obtain about 70 mg of purified protein per liter of bacterial growth. Considering the potential application of this new chimeric protein, our aim is to develop an uncomplicated, large scale, chromatography-free purification protocol with a shorter time frame, mainly based on crossflow ultrafiltration, starting from fermented bacterial paste.

Preliminary protein expression experiments were performed at three different IPTG concentrations and at different induction times and temperatures. The best condition was found to be 0.5 mM IPTG at 25 °C for 16 h. Customized large scale protein fermentation was then performed using these parameters. In Fig. 1 we report the SDS-PAGE of cell lysates. In this experimental condition, HumFt migrates as a monomer of the expected 20 kDa molecular weight. Lane 5 shows the high level of protein expression in the soluble fraction.

Large scale purification protocol was set up on the soluble fraction obtained after sonication of 100 g of bacterial paste grown under fermentative conditions. In this case, the amount of soluble HumFt was even higher of the one obtained growing the bacteria in flask. The SDS-PAGE in Fig. 2 shows the protein enrichment through the different steps
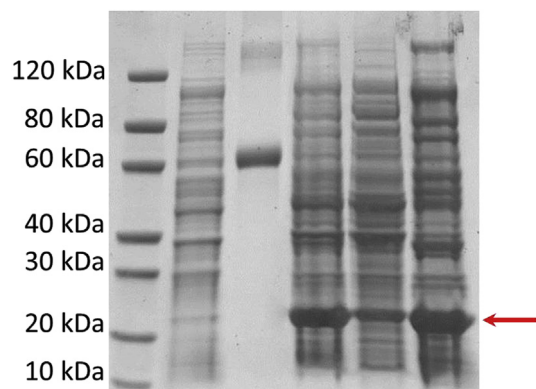

Fig. 1. Protein expression analyzed by SDS-PAGE. Lane 1: Protein Marker; lane 2: cell lysate without induction; lane 3: BSA (2 μg); lane 4: cell lysate induced by 0.5 mM IPTG at 25 °C for 16 h; lane 5: supernatant of cell lysate induced by 0.5 mM IPTG at 25 °C for 16 h; lane 6: pellet of cell lysate induced by 0.5 mM IPTG at 25 °C for 16 h. The arrow corresponds to HumFt monomer molecular weight.
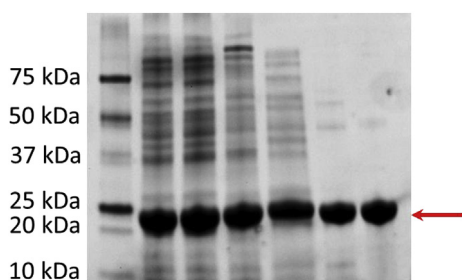
Fig. 2. Protein purification steps analyzed by SDS-PAGE. Lane 1: Protein Marker; lane 2: sonication supernatant; lane 3: 20% ammonium sulfate supernatant; lane 4: soluble fraction after heat treatment at 62 °C; lane 5: soluble fraction after heat treatment at 72 °C; lane 6: sample filtered using Celpure® C300 diatomaceous earth; lane 7: sample filtered through Vivaflow 200 module with a cutoff of 100 kDa.

Fig. 3. DNA removal followed by UV absorption spectroscopy. Continuous line: sample before the ultrafiltration step; dashed line: retained fraction; dotted line: filtered fraction.

of the procedure. In the first step, 20% ammonium sulfate has been used to precipitate some proteins, keeping HumFt in solution. A further purification was achieved by two sequential heating steps at 62 and 78 °C. Ferritins are, generally, thermostable proteins and HumFt displays an even higher thermostability as most of its sequence belongs to a thermophilic bacterium. These steps efficiently remove protein contaminants (including DNAse), but obviously fail to remove lipid micelles and DNA. Lipid micelles have been easily eliminated by a filtration step using highly pure diatomaceous earth, as proved by the overall clarification of the sample. HumFt does not interact with the diatomaceous earth and is fully recovered after the filtration. Other contaminants, instead, are retained by diatomaceous earth, leading to a further purification of the sample (Fig. 2, lane 6).

One of the major problems in the purification of recombinant ferritins is represented by bacterial DNA cross-contamination. DNA has to be removed in view of *in vitro* or *in vivo* experiments. Moreover, in the case of HumFt, we observed that it directly affects the quaternary structure of the protein. In the previously published protocol DNA removal was achieved by DNAse treatment followed by a gel filtration on a HiLoad 26/600 Superdex 200 pg column. This expensive and time consuming step can indeed be avoided by the combined use of DNAse coupled to crossflow ultrafiltration. This purification procedure has a great advantage as it can be used in concentration and diafiltration modes: in the first case, ferritin (480 kDa) is retained by the membrane (100 kDa cutoff), the buffer passes through, and the protein concentration increases over time; in the second case, the protein is concentrated to remove the excess of the starting buffer, then diluted with the new buffer and concentrated again. This process can be repeated until the buffer is completely exchanged.

To further purify ferritin-enriched solution, 1 L sample was concentrated ten times at a flow of 30–40 mL/min (transmembrane pressure: 1.5 bar), then diluted with 1 L of washing buffer for the diafiltration step, keeping the recirculation rate in the range of 30–40 mL/min. During this step, DNA was removed as proven by comparing the absorption spectra of filtered and unfiltered fractions. As shown in Fig. 3, compared to the spectrum obtained prior the ultrafiltration step, the retained fraction, enriched in ferritin is DNA free, displays a maximum absorption at 280 nm.

Once the buffer was completely exchanged by means of the diafiltration process, ferritin was concentrated to a final volume of 100 mL (20 mg/mL). HP-SEC performed on samples before and after the diafiltration step shows that the total protein content doesn't change at all. Ferritin is not detected in the permeate suggesting an about total protein recovery. The overall crossflow ultrafiltration process took about 1.5 h.

A further advantage is that the filtration modules can be regenerated and reused a number of times depending on the source and quality of the sample. In our experimental setting the filtration
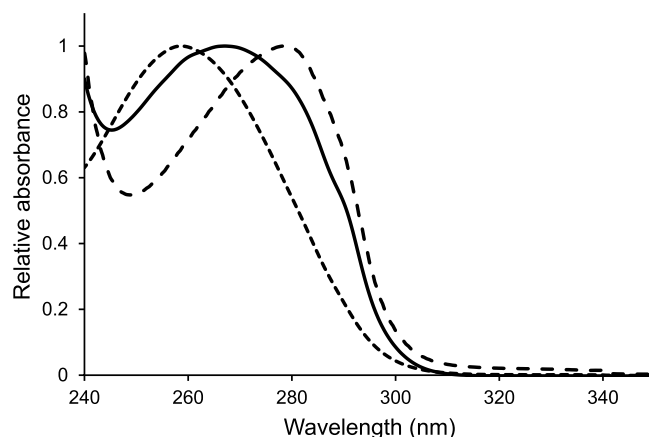
performance was kept up to 10 times.

This protocol can be indeed easily extended to all recombinant ferritins and most high molecular weight (> 100 kDa) proteins and it can be further scaled-up connecting several crossflow filtration modules in series and/or in parallel according to the specific needs.

One of the main challenges in large scale recombinant ferritin purification is achieving the complete DNA removal. This is a critical step for all proteins that are going to be used for *in vivo* applications and especially for ferritins that strongly interact with nucleic acids [23]. In the specific case of humanized ferritin, DNA traces could affect assembly/disassembly equilibrium. In fact, when HumFt is purified with conventional chromatographic methods [20], the very small amount of DNA co-eluting with the protein does not allow a complete quaternary assembly. HPLC-SEC analysis shows that up to 20% of ferritin is not assembled and elutes as a dimer (Fig. 4). When DNA is completely removed using the purification protocol here described, the protein elutes as a single peak corresponding to the 24mer (Fig. 4). As HumFt has been developed as a nanocarrier for drug delivery, the full control of the aggregation state is of paramount importance in order to obtain a highly efficient cargo system.
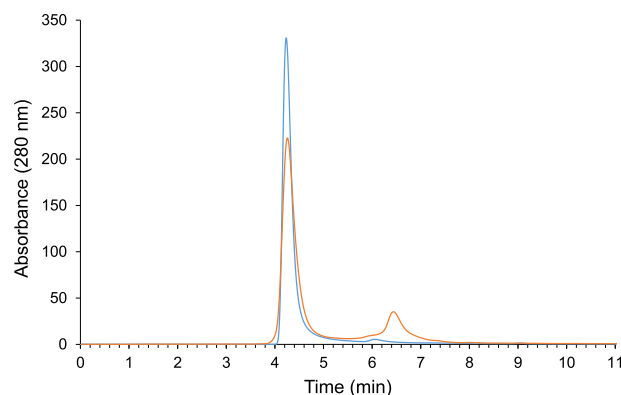

Fig. 4. HP-SEC of purified HumFt. Elution profile of HumFt followed at 280 nm. Red line: protein purified by means of HiLoad 26/600 Superdex 200 pg column. Blue line: protein purified by crossflow ultrafiltration. HumFt 24mer retention time: 4.22 min; HumFt dimer retention time: 6.31 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusions

In the present paper we describe a large scale, chromatography free protocol for the purification of a recombinant ferritin. To the best of our knowledge, this is the first report involving the use of diatomaceous earth filter aid coupled to crossflow ultrafiltration for the purification of a ferritin. The current procedure can easily remove contaminant proteins as well as DNA molecules in the absence of expensive and time consuming chromatographic steps. The whole procedure takes about one working day compared to the two/three days required for a typical purification protocol involving chromatographic steps. In addition, the use of crossflow ultrafiltration modules significantly reduces the overall purification costs, as they are cheaper than basic chromatography tools and can be extensively reused.

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