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Purification and on-column refolding of EGFP overexpressed as inclusion bodies in *Escherichia coli* with expanded bed anion exchange chromatography

C. Cabanne*, A.M. Noubhani, A. Hocquellet, F. Dole, W. Dieryck, X. Santarelli

Ecole Supérieure de Technologie des Biomolécules de Bordeaux (ESTBB), Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France

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Abstract

The enhanced green fluorescent protein (EGFP) was over-expressed in *Escherichia coli* as inclusion bodies to increase its quantity and to facilitate its purification. Insoluble EGFP has been purified on Q Hyper Z matrix by expanded bed adsorption after solubilization in 8 M urea. The adsorption was made in expanded bed mode to avoid centrifugation. EBA-column refolding was done by elimination of urea and elution with NaCl. The EGFP was obtained as a highly purified soluble form with similar behavior in fluorescence and electrophoresis as native EGFP.

Keywords: Expanded bed adsorption; EGFP; Purification; Chromatography; Refolding; Inclusion bodies

1. Introduction

Since the cloning of its gene from the jellyfish *Aequorea victoria* [1], the green fluorescent protein (GFP) has been widely used in cellular biology [2,3]. Its fluorescent properties have been changed by genetic engineering, leading to several mutants, especially enhanced GFP (EGFP) which has red-shifted excitation spectra (maximal excitation peak at 490 nm) and fluoresces (at 510 nm) 35-fold more brightly than wild-type GFP [4]. EGFP is easily quantified by fluorometric assay. The need to use large quantities of EGFP in our laboratory and its high market cost led us to develop its production and purification on a large scale. Recently, another mutant GFPuv was purified using salt-promoted IMAC [5], but high saline concentrations and pH gradient were required to elute protein with good recovery. Although a poly-Histidine tag was used in the past to purify EGFP [6,7], two-step purification was necessary to obtain pure EGFP. Some authors have attempted to purify GFP using the hydrophobic properties of n HPLC size exclusion chromatography [8], chromatofocusing with a pH gradient composed of multiple stepwise fronts [9], multi-step purification procedures, including precipitation of DNA, ammonium sulfate precipitation of GFP, size exclusion chromatography and concentration by hydrophobic chromatography or ultrafiltration, or using polymer to absorb water [10] or organic extraction [11].

These traditional techniques are limited with recombinant proteins by intrinsic problem of aggregation [12]. Indeed most of the recombinant proteins are overexpressed as inclusion bodies. The challenge is to convert the inactive and insoluble inclusion bodies protein aggregates into soluble, correctly folded biologically active products [13]. Mannen et al. [14] showed for the first time an expanded bed protein refolding using a solid phase artificial chaperone. They used the artificial chaperone assisted refolding method (ACA) and the expanded bed technique providing a novel industrial-

* Corresponding author. Tel.: +33 5 57 57 17 13; fax: +33 5 57 57 17 11.
E-mail address: charlotte.cabanne@estbb.u-bordeaux2.fr (C. Cabanne).

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scale protein refolding system. Cho et al. [12] have also proposed an expanded bed adsorption (EBA) mediated refolding process of a fusion protein. Exploiting advantages of expanded bed adsorption chromatography, which is an alternative to traditional clarification (centrifugation, tangential micro- and ultrafiltration and the first chromatography step [15–17]), they could provide higher yield with much less aggregation reducing the number of the renaturation process steps.

We overexpressed the recombinant EGFP as inclusion bodies to increase the quantity of recovered and to facilitate purification. The inclusion bodies were dissolved by urea and then the unfolded recombinant EGFP was allowed to adsorb on the chromatographic support. Our on-column purification and refolding procedure including anion exchange expanded bed technology was done in one step. The incentive of this approach was to obtain a one step purification of high quantity of EGFP suitable for automation.

2. Experimental

2.1. Instruments

The bioreactor was the Bioflow 3000 from New Brunswick Scientific (Edison, NJ, USA). The chromatographic system used throughout this study was the Biopilot chromatography system from Amersham Biosciences (Saclay, France). The data were collected and evaluated using the Unicorn director data system. The ultrasonic homogenizer Vibracell 72412, 600 W model was from Bioblock (Illkirch, France). The electrophoresis apparatus Mini-Protean II, electroporator Gene Pulser II and image analysis Gel Doc 2000 were from BioRad (Ivry-sur-Seine, France). For recovery studies, we used a Lambda Bio spectrophotometer (Perkin Elmer, Courtaboeuf, France) to measure absorbance at 595 and 600 nm. The fluorescence assays were performed with the luminescence spectrometer LS50B (Perkin Elmer).

2.2. Chemicals

pET 15b (plasmid for expression) and the *Escherichia coli* C41, which is lysogenic for bacteriophage DE3, were from Avidis (Saint-Beauzit, France). pEGFP was from Clontech Laboratories (Palo Alto, CA, USA). All salts were from Sigma (l'Isle d'Abeau Chesnes, France), Luria–Bertani (LB) culture media for the growth of *E. coli* and IPTG (Isopropyl β-D-thio-galactopyranoside) were from Invitrogen (Groningen, The Netherlands). *E. coli* JM109 was from New England Biolabs (Beverly, USA). DEAE Streamline and the Streamline 25 column were from Amersham Biosciences (Saclay, France). Q Hyper Z was from Biosepra (Cergy-Saint-Cristophe, France). All salts were HLPC grade and the buffers were filtered through a 0.22-μm membrane filter.

2.3. Preparation of cellular extract containing EGFP

2.3.1. Cloning and expression

A 0.72-kb fragment corresponding to the entire EGFP coding sequence was obtained by polymerase chain reaction (PCR) using the plasmid pEGFP as template. Cloning was done as previously described [7]. The PCR product was inserted into the pET 15b plasmid to create the EGFP construction, which was expressed in *E. coli* C41 according to the manufacturer's instructions.

2.3.2. Cell culture

The cells of *E. coli* C41 strain transfected by the plasmid pET 15b containing the EGFP construction were grown at 37 °C in a bioreactor in 1.5 l of LB medium (yeast extract 5 g/l, bactopeptone 10 g/l, NaCl 5 g/l, glucose 1 g/l) with ampicillin (100 μg/ml) and tetracycline (10 μg/ml). When the absorbance at 600 nm was 0.6 (1.7×10^8 cells/ml), IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 0.3 mM and the temperature of the culture was decreased to 30 °C. After 15 h of protein induction, the culture medium was refrigerated and centrifuged.

2.3.3. Ultrasonic homogenization

Cell pellets containing inclusion bodies were resuspended in 40 ml cold 20 mM sodium bicarbonate, pH 9.0, and the ultrasonic treatment of 40 ml of cell culture was performed at 20 kHz with a 13 mm probe. Four 45 in. pulses with 1 min in ice between each pulse were performed.

2.3.4. Solubilization of inclusion bodies

The ultrasonic homogenate was diluted with 360 ml of 8 M urea, 20 mM sodium bicarbonate pH 9.0 and agitated 2 h at 15 °C. Then, the homogenate was directly used for expanded bed anion exchange adsorption.

2.4. Chromatographic procedure

2.4.1. Measuring degree of expansion

The degree of expansion was calculated from the ratio of expanded bed height (L) to sedimented bed height (L_0). To study the expansion versus flow-rate, increments of 0.5 ml/min of the flow-rate were used from 0.5 ml/min until the flow rate necessary to obtain a twofold degree of expansion.

2.4.2. Influence of equilibration buffer and sample on degree of expansion

Before purification, we compared two expanded bed adsorption matrices: DEAE Streamline and Q Hyper Z. The flow-rate was compared for the same degree of expansion. The influence of high concentrations of urea and sample on the expansion was also studied. The matrix allowing the higher flow rate was chosen for the subsequent purification step.

2.4.3. Purification of recombinant EGFP from insoluble inclusion bodies

Anion exchange expanded bed adsorption was performed with Q Hyper Z (90 ml, 15.5 cm sedimented gel) packed in the Streamline 25 column. The column was linked to a Biopilot workstation. Equilibration/expansion was performed with 8 M urea, 20 mM sodium bicarbonate, pH 9.0 buffer at 44 ml/min to obtain a twofold degree of expansion. The homogenate was applied at 44 ml/min, followed by washing with the equilibration buffer until UV baseline was reached. Then the pump was turned off and the bed sedimented. Next, the adaptor was moved down towards the sedimented bed surface. After a run of two volumes (sedimented gel) of equilibration buffer, refolding was performed with 10 column volumes of 20 mM sodium bicarbonate, pH 9.0 buffer at a flow velocity of 44 ml/min using a downward flow in the sedimented bed mode. Then elution was performed in two steps, 150 mM NaCl, 20 mM sodium bicarbonate pH 9.0 for the elution of EGFP, and 1 M NaCl, 50 mM Tris–HCl, pH 8 for the elution of contaminants, and run at 44 ml/min.

2.5. Analytical procedures

2.5.1. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18] using a Mini-protean II apparatus and a Tris–Glycine–SDS buffer were used to monitor the purification during the chromatographic procedures. Electrophoresis was performed for 30 min at 200 V using 12% polyacrylamide gels. Detection was performed with Coomassie brilliant blue R250.

2.5.2. Protein concentration

The protein concentration was estimated by determining the total protein concentration using Coomassie blue [19] with bovine serum albumin as standard.

2.5.3. Fluorometric assays

Fluorescence measurements of native (0.296 μM) and refolded (0.271 μM) EGFP were performed in a Perkin Elmer luminescence spectrometer (cuvette 10 mm × 4 mm, 1 ml). We recorded the excitation and emission spectra of the both samples.

3. Results and discussion

3.1. Influence of equilibration buffer and sample on degree of expansion

Before purification, we have compared two matrices of expanded bed adsorption: DEAE Streamline and Q Hyper Z. Two buffers were evaluated, 20 mM sodium bicarbonate, pH 9.0 buffer and 8 M urea, 20 mM sodium bicarbonate, pH 9.0 buffer. The flow-rate was compared for twofold degree of expansion.

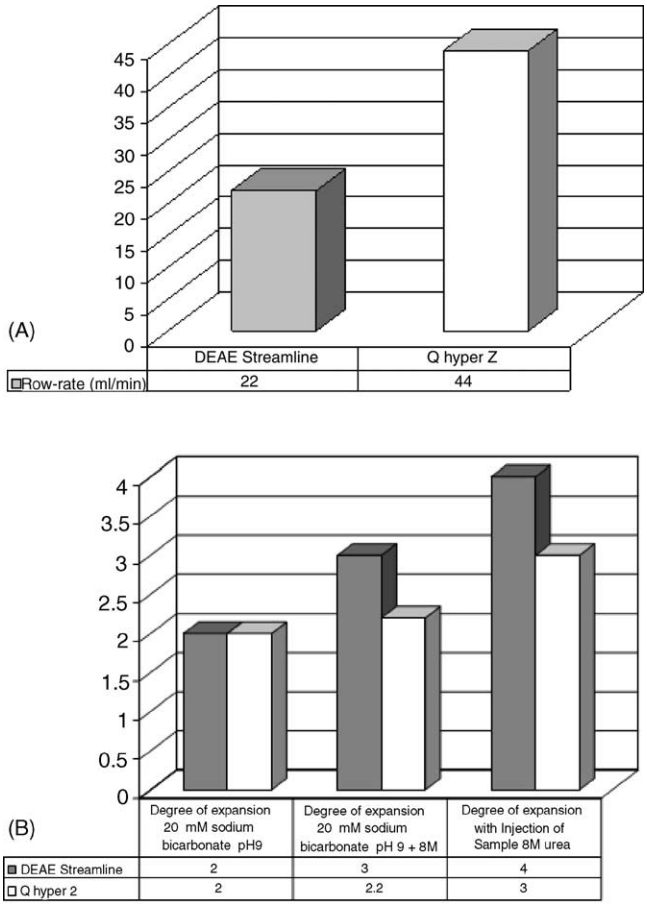

Fig. 1. (A) Flow rate necessary for an expansion degree of 2 with DEAE Streamline and Q Hyper Z matrices. Column: Streamline 25 (90 ml for the two matrices). Buffer: 20 mM sodium bicarbonate pH 9. (B) Influence of fluid on expansion degree with DEAE Streamline and Q Hyper Z. Column: Streamline 25 (90 ml for the two matrices); (a) 20 mM sodium bicarbonate pH 9; (b) 8 M urea, 20 mM sodium bicarbonate pH 9; (c) crude extract, 8 M urea, 20 mM sodium bicarbonate pH 9.

Fig. 1A shows that with 20 mM sodium bicarbonate pH 9 for a twofold degree of expansion, the Q Hyper Z matrix allowed a flow-rate twice that of DEAE Streamline. Fig. 1B shows that 8 M urea had less influence on Q Hyper Z than DEAE Streamline. The influence was similar with crude extract in presence of 8 M urea. Therefore, for purification we used the Q Hyper Z matrix, which allowed a high flow rate and gave satisfactory performance in the presence of 8 M urea. Moreover, in a previous study, we showed that this matrix was the most efficient to purify soluble EGFP from crude feedstock [20].

3.2. Purification of insoluble EGFP from inclusion bodies by anion exchange expanded bed chromatography

Equilibration/expansion was performed with 20 mM sodium bicarbonate, pH 9.0 buffer at 44 ml/min upward flow to obtained twofold degree of expansion. Then, equilibration was performed with 8 M urea, 20 mM sodium bicarbonate,

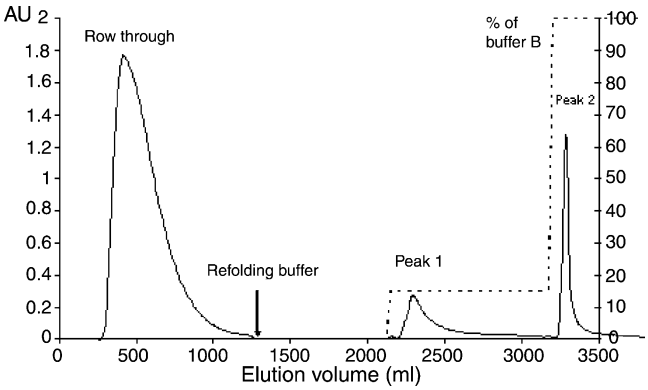

Fig. 2. Purification of insoluble EGFP with anion exchange expanded bed adsorption. Column: Q Hyper Z (90 ml of gel). Sample: unclarified ultrasonic cell homogenate with inclusion bodies in 8 M urea, 20 mM sodium bicarbonate pH 9. Equilibration buffer: 8 M urea, 20 mM sodium bicarbonate pH 9. Refolding buffer: 20 mM sodium bicarbonate pH 9. Elution buffer: 1 M NaCl, 20 mM sodium bicarbonate pH 9. Detection at 280 nm; flow-rate: 44 ml/min. Flow-through: no retained fraction; Peak 1: 150 mM NaCl eluted fraction; Peak 2: 1 M NaCl eluted fraction.

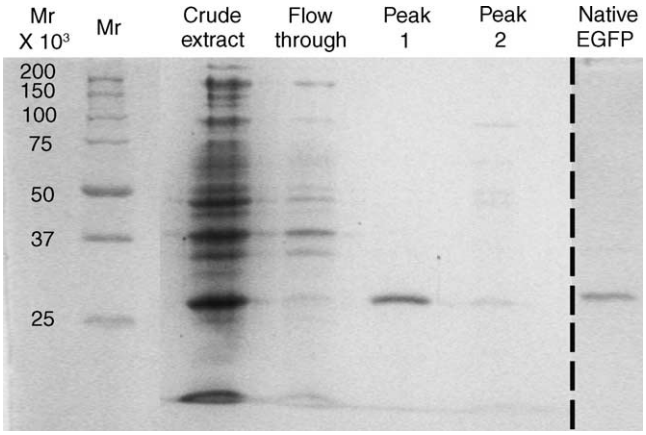

Fig. 3. SDS-PAGE analysis of fraction from purification of insoluble EGFP. Electrophoresis was performed using 12% polyacrylamide gels. Detection was performed with Coomassie brilliant blue R250. Crude extract: unclarified ultrasonic cell homogenate with inclusion bodies in 8 M urea, 20 mM sodium bicarbonate pH 9. Flow-through: no retained fraction; Peak 1: 150 mM NaCl eluted fraction; Peak 2: 1 M NaCl eluted fraction.

pH 9.0 buffer, giving a 2.2-fold degree of expansion. The inclusion bodies solubilized in 8 M urea (400 ml) were applied at 44 ml/min and gave a threefold degree of expansion. Injection of the sample was followed by washing with 8 M urea, 20 mM sodium bicarbonate pH 9.0 buffer until UV baseline was reached. Then the gel bed was sedimented and a run of two volumes (sedimented gel) of equilibration buffer was performed. Urea was eliminated with 10 column volumes of 20 mM sodium bicarbonate, pH 9.0 buffer at a flow velocity of 44 ml/min using a downward flow in the sedimented bed mode.

Next, the elution was performed in two steps, 150 mM NaCl, 20 mM sodium bicarbonate pH 9.0 for the elution of EGFP, and 1 M NaCl, 20 mM sodium bicarbonate pH 8 for the elution of contaminants, and was run at 44 ml/min (Fig. 2). All fractions were analyzed by total protein content and fluorescence analysis. The purification results are shown in Table 1. The crude extract (1.3 g of total protein) containing 27% of EGFP was applied onto the column, 68% of proteins passed into the flow-through, and the EGFP estimated by fluorescence was about 30 μg/ml. Most EGFP was captured by the Q Hyper Z matrix. Peak 1 contains 95% of EGFP with 90% of recovery and a purification factor of 3.5. The recovery and purification fold values are higher than those obtained in anion exchange EBA purification of soluble EGFP [20] but similar as those obtained in on line purification with immobilized metal affinity chromatography EBA purification of soluble EGFP [21]. However, purification with IMAC EBA required an additional step to obtain a single band on SDS PAGE [7]. The production of EGFP as inclusion bodies allowed us to reduce the volume culture for the same quantity of protein and then the time of process. Moreover, the use of anion exchange allowed us to avoid IMAC and therefore the use of heavy metals, which are toxic.

All fractions were analyzed by SDS-PAGE (Fig. 3). The lane containing the flow-through shows a low level of EGFP, which is in accordance with the results of fluorescence shown in Table 1. It seems that this EGFP corresponds to insufficient refolded EGFP. Indeed without urea treatment, all the EGFP would have passed in the flow-through (data not shown). The native EGFP from previous purification is shown as reference [20]. The molecular mass of refolded EGFP seemed to correspond to that of the native EGFP.

The folding of soluble EGFP obtained was evaluated by fluorescence analysis. We obtained the same excitation wavelength (490 nm) and emission wavelength (509 nm) maxima for the native (0.296 μM) and refolded (0.271 μM) EGFP (Fig. 4). Moreover, the shape of the spectra was the same for the both samples. The origin of the intensity difference is the concentration of samples. So the chromophore in the refolded EGFP has the same configuration.

Table 1
Purification of EGFP on EBA with the Q Hyper Z matrix

	Volume (ml)	Protein (mg/ml)	Total protein (mg)	EGFP (mg/ml)	Total EGFP (mg)	Specific fluorescence (mg EGFP/mg total protein)	Recovery (%)	Purification (fold)
Crude extract	400	3.30	1320	0.90	360.0	0.27		
Flow-through	1050	0.86	903	0.03	31.5	0.03	8.8	0.1
Peak 1	260	1.32	343	1.25	325.0	0.95	90.3	3.5
Peak 2	230	0.07	16	0.01	1.4	0.09	0.4	0.3

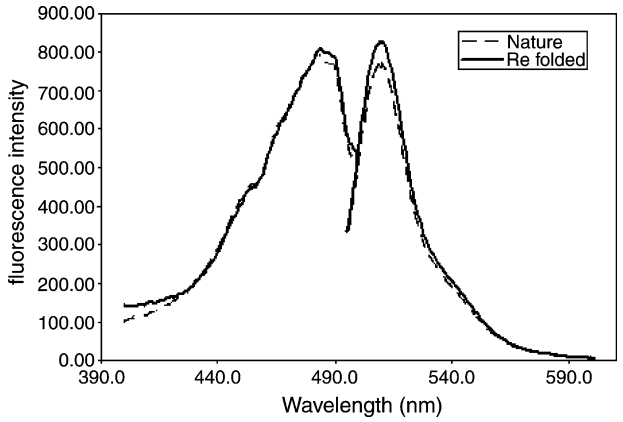

Fig. 4. Fluorescence analysis with the Perkin Elmer luminescence spectrometer. Excitation and emission spectra of the native (0.296 μM) and refolded (0.271 μM) EGFP (cuvette 10 mm × 4 mm, 1 ml). The maximum of excitation was obtained at 490 nm and the maximum of emission at 509 nm.

4. Conclusion

In this paper, we show that the high density of Q Hyper Z beads allows efficient processing of feedstocks at very high viscosity (urea 8 M) and high flow rate (44 ml/min). Insoluble EGFP could be purified after solubilization in 8 M urea and adsorption in expanded bed mode to avoid centrifugation. After elimination of urea on the column and elution with NaCl, the EGFP was obtained as a highly purified soluble form with similar behavior in fluorescence and electrophoresis as native EGFP. The results of one-step protein purification and on-column refolding of EGFP with anion exchange expanded bed chromatography demonstrate that it is a promising process allowing automation.

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