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Cloning, expression and two-step purification of recombinant His-tag enhanced green fluorescent protein over-expressed in *Escherichia coli*

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Abstract

In this report, we describe a two-step chromatographic procedure for the purification of His-tag EGFP by immobilized metal affinity expanded bed adsorption (IMAEBA) as the capture step and size exclusion chromatography as the polishing step. The use of proteins including a histidine-tag facilitates their subsequent purification after expression in many microorganisms. This meets the needs of scientific researchers as well as industrialists in purifying recombinant proteins. The procedure described allowed the obtention of 230 mg pure EGFP from 1 l simple batch culture with a recovery of 90%.

Keywords: Cloning; Expression; Purification; *Escherichia coli*; Enhanced green fluorescent protein

1. Introduction

Since the cloning of its gene starting 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 EGFP (enhanced GFP) 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. Therefore, we over-expressed recombinant EGFP as a His-tag (histidine-tag) protein in *E. coli* to facilitate purification [5,6]. The His-tag allowed us to use immobilized metal affinity chromatography (IMAC) [7–9]. Recently another mutant GFPuv was purified using salt-promoted IMAC [10], but high saline concentrations and pH gradient were used to elute protein with good recovery. Although His-tag was used in the past to purify EGFP [11], purification was not satisfactory (80%) and no recovery was described. Some authors attended to purify GFP using hydrophobic properties of an HPLC size exclusion chromatography [12], chromatofocusing with a pH gradient composed of multiple stepwise fronts [13], multi-steps purification procedure, including precipitation of DNA, ammonium sulfate precipitation of GFP, size exclusion chromatography and concentration by hydrophobic chromatography or ultrafiltration or using polymer to

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absorb water [14] or organic extraction [15]. Our two-step procedure including immobilized metal affinity expanded bed technology, an alternative to traditional clarification (centrifugation, tangential micro- and ultrafiltration and the first chromatography step) [16–18], makes it possible to process directly large volumes of crude feedstock with fast adsorption of the target molecule early in the process to prevent its degradation. A final polishing step is used to obtain pure EGFP with a high degree of purity.

2. Experimental

2.1. Instruments

The chromatographic system used throughout this study was the Biopilot and AKTA explorer workstation from Amersham Bioscience (Saclay, France). The data were collected and evaluated using the Unicorn Data system. The ultrasonic homogenizer Vibracell 72412 from Bioblock (Illkirch, France) was used with a 19-mm probe. For recovery studies, we used a Lambda Bio UV spectrophotometer (Perkin-Elmer, Courtaboeuf, France).

The fluorescence assays were performed with the Versafluor fluorometer from BioRad (Marnes-la-Coquette, France). The proteins were concentrated using the Millipore Labscale TFF system from Millipore (Saint-Quentin-en-Yvelines, France). The electrophoresis apparatus used was the Mini-Protean II from Bio-Rad (Marnes-la-Coquette, France).

2.2. Chemicals

Chelating Streamline, Superdex 75 prep grad, Streamline 25 column and XK 16/70 column were from Amersham Bioscience (Saclay, France). Ultrafiltration membrane Biomax 10 (50 cm^2) was from Millipore (Saint-Quentin-en-Yvelines, France). pET 15b (plasmid for expression) and the *E. coli* NOVABLUE (DE3), which is lysogenic for bacteriophage DE3, were from Novagen (Madison, WI, USA). pEGFP was from Clontech Laboratories (Palo Alto, CA, USA). All salts were from Sigma (l'Isle d'Abeau Chesnes, France), and the buffers were filtered through a 0.22-μm membrane filter.

2.3. 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. The 5′ primer contained a 10-nucleotide linker with an *Nde*I (*Neisseria denitrificans*) restriction site to facilitate the in-frame subcloning with the tag domain of pET 15b and the codons for the first six N-terminal residues. The 3′ primer contained an 8-nucleotide linker with a *Bam*HI (*Bacillus amyloliquefaciens* H) restriction site, a stop codon and five codons for the C-terminal extremity. The *Nde*I/*Bam*HI digested PCR product was inserted into the *Nde*I/*Bam*HI linearized pET 15b plasmid to create the EGFP His-tag construction (Fig. 1). The resulting EGFP His-tag protein was expressed in *E. coli* NOVABLUE (DE3) according to the manufacturer's instructions.

2.4. Cell culture

The cells of *E. coli* NOVABLUE (DE3) strain transfected by the plasmid pET 15b containing the EGFP His-tag construction were cultured 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 amphicillin (100 μg/ml) and tetracycline (10 μg/ml). When the absorbance at 600 nm was 0.6 ($1.7 \cdot 10^8$ cells/ml), IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 0.3 m*M* and the temperature of the culture was decreased to 30 °C. After 15 h of protein induction, the culture medium was refrigerated and treated with an ultrasonic probe to break the cell walls.

2.5. Ultrasonic homogenization

The ultrasonic treatment of 500 ml of cell culture was performed at 20 kHz with a 19-mm probe. Four 2-min pulses with 2 min in ice between each pulse were performed.

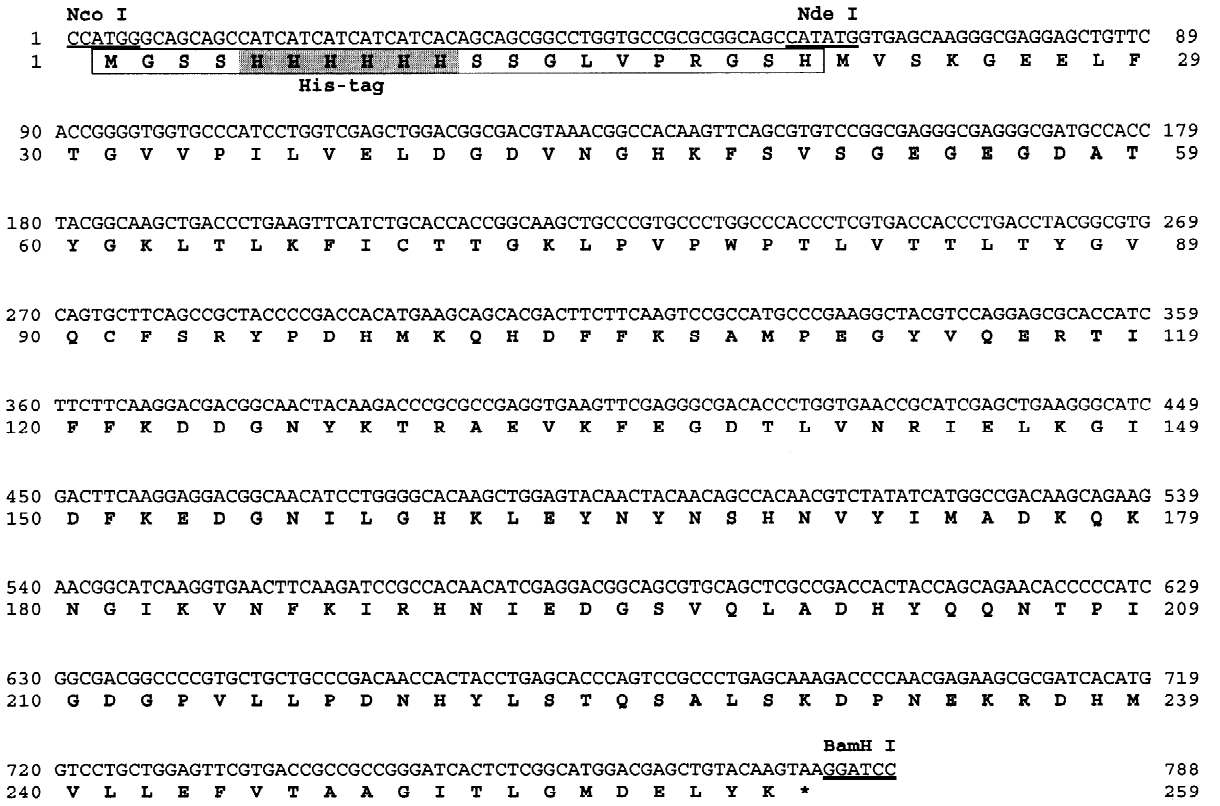

Fig. 1. Sequence of the gene fusion coding for the His-tag EGFP. This sequence corresponds to the *Nco*I (*Nocardia corallina*)/*Bam*HI DNA fragment from the 6.097-kb plasmid obtained after insertion of the *Nde*I/*Bam*HI digested PCR product into the *Nde*I/*Bam*HI linearized pET15b plasmid. The N-terminal extension of EGFP is boxed. The His-tag sequence is colored grey. Restriction sites of *Nco*I, *Nde*I and *Bam*HI are underlined. The stop codon is indicated with a star.

2.6. Immobilized metal affinity chromatography

An IMAEBA (immobilized metal affinity expanded bed adsorption) was performed with Chelating Streamline (90 ml, 15 cm sedimented gel) packed in the Streamline 25 column and charged in metal by 100 ml of 0.1 *M* $NiSO_4$ in water. The column was linked to a Biopilot workstation. Equilibration/expansion was performed with 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8 buffer at 30 ml/min to obtained 3 degrees of expansion that is the maximum recommended for this technology and the size of the column used. The unclarifed feedstock was applied at 30 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, elution was performed with 100 m*M* imidazole, 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8 buffer at a flow velocity of 15 ml/min using a downward flow in the sedimented bed mode. The washing buffer, i.e. 500 m*M* imidazole, 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8, was run at 15 ml/min. The eluted peak was concentrated to 15 ml with Millipore Labscale TFF system and injected into the size exclusion chromatography.

2.7. Size exclusion chromatography

A size exclusion chromatography was performed with the Superdex 75 prep grad column packed in the XK 16/70 column according to the manufactur-

er's recommendations (1 ml/min for 3 h and 4 ml/min for 45 min with distilled water). The column equilibration was performed at 1 ml/min with 5 column volumes of buffer until the baseline is stable. The equilibration buffer used was 150 m*M* NaCl, 50 m*M* sodium phosphate, pH 7 and the run was performed at 1 ml/min with 5 ml of sample. Three runs were performed.

2.8. Analytical procedures

2.8.1. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [19] using the mini-Protean II apparatus and a Tris–glycine buffer system was used to monitor purification during chromatography. The gels were run under reducing conditions with heat treatment of the samples (95 °C, 5 min) and electrophoresis was performed for 45 min at 200 V using 12% polyacrylamide gels. Detection was performed with Coomassie brilliant blue R250 staining.

2.8.2. Fluorometric assays

During all experiments, samples were centrifuged for 5 min at 5000 *g* and the EGFP concentration was estimated in supernatant by assessing fluorescence at 510 nm after excitation at 490 nm. A curve of reference fluorescence (rfu)/quantity of pure EGFP indicated the quantity of EGFP produced, i.e. free by ultrasonic treatment and then purified.

2.8.3. Protein concentration

The protein concentration was estimated by bicinchoninic acid methods [20] using bovine serum albumin as standard.

3. Results and discussion

In our construction, EGFP is a protein possessing a Histidine-tag, which is expressed under the dependence of the *lac z* operon. After induction it is not excreted in the culture medium. In order to achieve its purification, it was necessary to harvest the cells, to break them, and to perform different stages of centrifugation or filtration in order to eliminate the cellular remnants before moving to the purification steps. At least six treatment steps were needed before the purified product was obtained. The use of expanded bed technology allowed the number of steps to be decreased.

3.1. Ultrasonic homogenization

Ultrasonic treatment constitutes a major advantage during purification of proteins by the method of expanded bed adsorption, since the viscosity of the culture medium does not increase, even when the biomass is large. The increase in the viscosity of the feedstock generally induces an aggregation of the chromatographic gel beads that stick to the top filter of the column and raise the piston. Ultrasonic treatment induces breaks at the level of the nucleic acids that are responsible for modifying the viscosity of the culture medium when other techniques are used to break the cells [21–23].

3.2. Immobilized metal affinity expanded bed adsorption (IMAEBA)

Expansion, equilibration and washing were performed using a flow-rate of 30 ml/min, which resulted in a degree of expansion of 3. Application of the unclarified feedstock was performed at 30 l/min with a 2.5 degree of expansion due to fouling. After the ultrasonic treatment, about 95% of proteins were passed into the through-flow and EGFP was captured by the IMAEBA (Fig. 2). Table 1 shows that 94% of EGFP was in peak 1, 4% in peak 2 and 5% of EGFP was in the through-flow. The factor of purification was 19 after IMAEBA.

3.3. Size exclusion chromatography

The concentrated eluted IMAEBA peak was injected onto the Superdex 75 prep grad at 1 ml/min flow-rate (Fig. 3). The contaminants observed on the SDS–PAGE were eluted between 40 and 60 ml of elution volume and the EGFP was eluted at 70 ml. The factor of purification obtained was 23 with 90% of recovery.

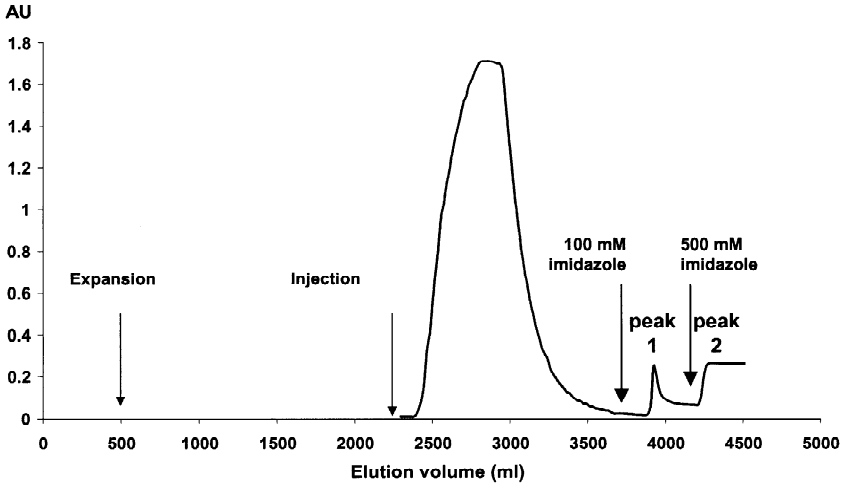

Fig. 2. Capture of recombinant EGFP with chelating Streamline. Column: Streamline 25 (90 ml of gel). Sample: unclarified and ultrasonic homogenate of cell culture: 500 ml; equilibration buffer: 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8; elution buffer: 0.5 *M* imidazole, 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8. Detection at 280 nm; flow-rate: 30 ml/min in expansion/equilibration, feed application and elution.

3.4. Analysis of proteins by SDS–PAGE

The analysis of the proteins contained in the different purification fractions showed (Fig. 4) that the EGFP His-tag had a relative mass (M_r) of about 26 000. On the track corresponding to peak 1 of IMAEBA there was a majority strip at (M_r) 26 000 and two minority strips at (M_r) 49 000 and 59 000. This confirmed the 85% purity of the protein. The track corresponding to peak 2 showed these three strips to be weakly stained. The track of the proteins from the non-retained fraction showed a weak marking of a strip at (M_r) 26 000 that could correspond to the 5% of EGFP not recovered in peak 1. The track from SEC showed a pure EGFP.

4. Conclusion

We have developed a two-step method for purifying a recombinant protein overexpressed in *E. coli* including ultrasonic treatment of the cell culture, IMAEBA and SEC. More than 115 mg of EGFP were obtained from 0.5 l cell culture (230 mg/l) while reducing the purification process to two steps.

The use of EGFP allowed us to detect its presence

Table 1
Purification EGFP from 0.5 l of cell culture

	Total protein (g)	Total EGFP (g)	EGFP recovery (%)	Purification (-fold)
Starting material	2.931	0.128	–	–
Non-retained IMAEBA	2.78	0.007	–	–
IMAEBA peak 1	0.140	0.120	94	19
IMAEBA peak 2	0.036	$4{\cdot}10^{-4}$	3	–
SEC	0.12	0.115	90	23

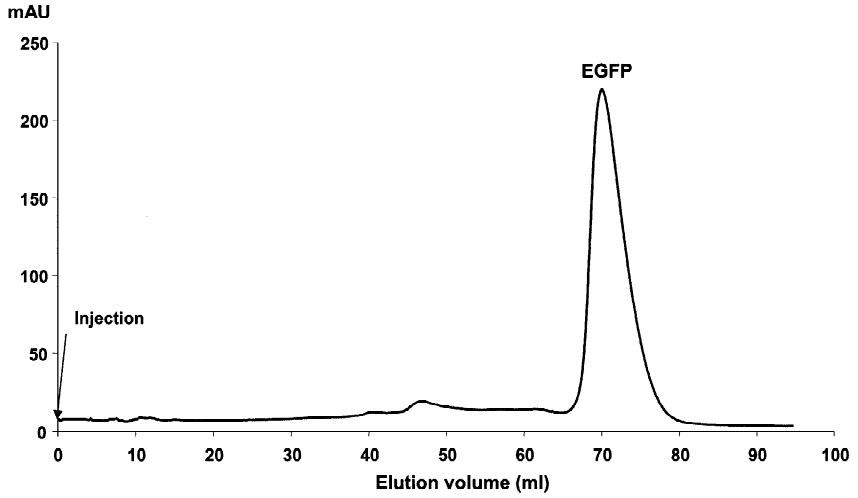

Fig. 3. Polishing step with Superdex 75 prep grad. Column: XK 16/70 (120 ml of gel). Sample: eluate of IMAEBA concentrated to 15 ml; sample volume: 5 ml. Buffer: 150 m*M* NaCl, 50 m*M* sodium phosphate pH 7. Detection at 280 nm; flow-rate: 1 ml/min.

easily throughout the experiments by fluorescence measurement. The use of expanded bed technology allowed the molecule of interest to cling very quickly to the IMAC support and the polishing step allowed purity to be increased with high recovery.

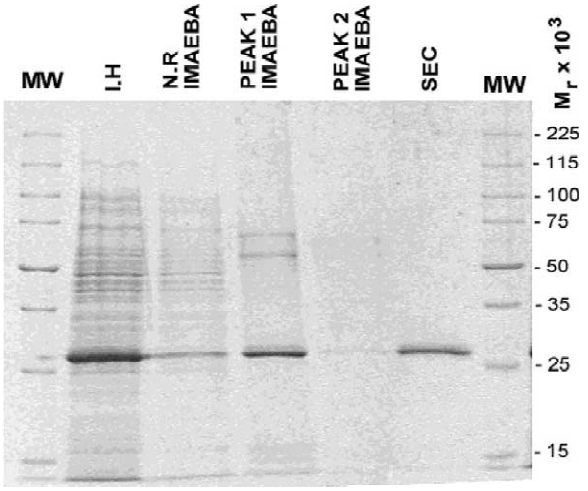

Fig. 4. Samples from the different EGFP purification steps were analyzed by SDS–PAGE electrophoresis. The proteins from the induced cell homogenate (IH), the non-retained fraction (NR IMAEBA), the peak 1 (peak1 IMAEBA) and the peak 2 (peak 2 IMAEBA) of IMAEBA and the eluted fraction of SEC (SEC) were loaded in each lane of the (12%) acrylamide gel. In each lane, 15 μg of proteins were loaded, except for the peak 2 of IMAEBA (8 μg) which was too diluted. At the end the electrophoresis the gel was Coomassie-stained.

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