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On-line purification of His-tag enhanced green fluorescent protein taken directly from a bioreactor by continuous ultrasonic homogenization coupled with immobilized metal affinity expanded bed adsorption

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Abstract

In this report, we describe a new process for the on-line purification of His-tag EGFP (enhanced green fluorescent protein) taken directly from a bioreactor by continuous ultrasonic homogenization coupled with immobilized metal affinity expanded bed adsorption (IMAEBA). 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 interested in purifying recombinant proteins. After evaluating the different flow-rates and ultrasonic probe sizes, the on-line purification was tested. After ultrasonic treatment, 70% of the cells were broken and 90% of free EGFP was recovered after IMAEBA. In our conditions, more than 450 mg of EGFP were obtained in 15 h. On-line bioreactor–ultrasonic probe–immobilized metal affinity expanded bed adsorption is a rapid automated technique for obtaining large quantities of pure EGFP.

Keywords: Immobilized metal affinity chromatography; Expanded bed adsorption; *Escherichia coli*; Enhanced green fluorescent protein; Proteins

1. Introduction

Expanded bed adsorption (EBA), an alternative to traditional clarification (centrifugation, tangential micro and ultrafiltration and the first chromatography step) [1–3], may be used to process directly large volumes of crude feedstock. The low processing time at the capture step just after fermentation is essential because the fast adsorption of the target molecule early in the process prevents its degradation. If the molecule of interest is intracellular, the cell must be destroyed to release the protein. Several techniques such as mechanical breakage (liquid and solid shear) and non-mechanical breakage (desiccation and lysis) are available [4]. While the increased use of EBA technology in processing downstream has improved the production of proteins, the on-line purification of

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protein taken directly from the bioreactor has been little investigated recently. The direct process integration of cell disruption and purification studied with yeast [5] does not provide good recovery (11%) and generates high viscosity. The present paper therefore describes on-line purification of recombinant protein over-expressed in *Escherichia coli* taken directly from a bioreactor.

The efficiency of on-line purification was assessed with enhanced green fluorescent protein (EGFP). Since the cloning of its gene that started from the jellyfish *Aequorea victoria* [6], the green fluorescent protein (GFP) has been widely used in cellular biology [7,8]. Its fluorescent properties have now been changed by genetic engineering to provide several mutants, especially EGFP (enhanced GFP) which has red-shifted excitation spectra (maximal excitation peak at 490 nm) and fluorescence (at 510 nm) 35-fold brighter than wild-type GFP [9]. We used the EGFP as an easily quantifiable protein by fluorometric assay to develop a new process of purification. We over-expressed the recombinant EGFP as a His-tag (histidine-tag) protein in *E. coli*. The His-tag made it possible to use immobilized metal affinity chromatography (IMAC) [10–14]. The use of IMAC with expanded bed adsorption was recently described for native [15] and recombinant His-tag protein [16,17].

The disruption technology applied to *E. coli* was ultrasonic homogenization used with a continuous flow cell. After optimizing the cell disruption of cells versus flow-rate, the on-line purification of EGFP directly from a bioreactor was performed and analyzed.

Recently, a company produced a new high power ''Pilotstation'' (2000 to 4000 W) able to treat a large volume reactor, with a flow-rate of about 6 l min^{-1}. Commercial applications of our new on-line purification process should now be developed.

2. Experimental

2.1. Instruments

The chromatographic system used throughout this study was the Biopilot workstation from Amersham Bioscience (Saclay, France). The data were collected and evaluated using the Unicorn Data system. The ultrasonic homogenizer Vibracell 72412, 600 W model from Bioblock (Illkirch, France) was used with a continuous flow cell and two probes were tested (13 and 19 mm). The volume of the ultrasonic flow cell was 48 ml with the 13 mm probe and 38 ml with the 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 Bio-Rad (Ivry sur Seine, France). The proteins were concentrated using the Vivaspin concentrator from Sartorius (Palaiseau, France). The electrophoresis apparatus used was the Mini-Protean II from Bio-Rad.

2.2. Chemicals

Streamline Chelating and Streamline 25 column were from Amersham Bioscience. pET 15b (plasmid for expression) and the *E. coli* Novablue (DE3) which is lysogenic for bacteriophage DE3 were from Novagen (Madison, WI). pEGFP was from Clontech Labs (Palo Alto, CA). 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-kilobase pair (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 six first 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. 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^{-1}, bactopeptone 10 g l^{-1}, NaCl 5 g l^{-1}, glucose 1 g l^{-1}) with ampicillin (100 μg ml^{-1}) and tetracycline (10 μg ml^{-1}). When the absorbance at 600 nm was 0.6 (1.7×10^{8} cells ml^{-1}), 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. Continuous ultrasonic homogenization

The feed-stream was directly injected by peristaltic pump from the bioreactor into the continuous ultrasonic flow cell. The ultrasonic treatments were performed at 20 kHz. The non-stop ultrasonic flow cell was permanently cooled by circulation of the cooling liquid from the cryostat to the jacket of the flow cell. The medium temperature was 20 °C in the exit of the ultrasonic probe. Several flow-rates and two probes were tested to optimize the ultrasonic homogenization.

A fraction of the culture medium before and after ultrasonic treatment was spread on Petri dishes containing LB agar+ampicillin. Monitoring of cell viability (viable cells after treatment/cells before treatment) provided an indication of the efficiency of the ultrasonic treatment.

2.6. Immobilized metal affinity expanded bed adsorption

An IMAC system using EBA technology was performed with the Streamline 25 column containing 93 ml of Streamline Chelating corresponding to a sedimented bed height of 19 cm.

The Streamline 25 column was linked to a Biopilot workstation, which in turn was linked to the continuous ultrasonic flow cell. The evaluation of bed stability was performed by visual inspection, by measuring the degree of expansion and by the number of theoretical plates according to the manufacturer's instructions. The buffer used during expansion/equilibration was 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8. The unclarified feedstock was applied just after ultrasonic homogenization directly onto the expanded bed, followed by washing with the equilibration buffer until UV baseline was reached. Then the pump was turned off and the bed was 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 at a flow velocity of 780 ml h^{-1} using a downward flow in the sedimented bed mode. The wash buffer, i.e. 500 m*M* imidazole, 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 8, was run at 780 ml h^{-1}.

2.7. Analytical procedures

2.7.1. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [18] 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 (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.

2.7.2. Fluorometric assays

During all experiments, samples were centrifuged for 5 min at 4620 *g* and the concentration of EGFP was estimated in the 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.

2.7.3. Protein concentration

The protein concentration was estimated by Bicinchonic acid methods [19] using bovine serum albumin as standard.

3. Results and discussion

3.1. Continuous ultrasonic homogenization

To develop an on-line system of EGFP purification, it was necessary to choose an ultrasonic probe and to define an optimal flux from the bioreactor via the ultrasonic probe toward the purification column. We tested two probes, one of 13 mm and the second of 19 mm.

At the end of every experiment, we tested the different flow-rates, corresponding to different durations of passage through the ultrasonic vibracell. The residence time of the fluid ranged from 1.8 to 9.6 min for the 13 mm probe, and 1.4 to 7.6 min for the 19 mm probe depending on the flow-rate used.

In the case of the 13 mm probe (Fig. 1) at 10 to 15 ml min^{-1} flow-rate, the free fluorescence was maximal and the corresponding viability was lowest (40%). In fact, this flow-rate allowed the elimination of 60% of the cells with a large increase in EGFP in the medium. The viability of the cells decreased quickly as the flow-rate increased up to 18 ml min^{-1}. Beyond this value, the duration of passage was low, the residence time was about 2.6 min, so cell viability increased thereafter.

The increase in EGFP in the medium was in agreement with our observation, i.e. the greater the cell mortality, the greater the fluorescence, and vice-versa.

We then tested the 19 mm probe (Fig. 2). Results were very similar to those for the 13 mm probe, i.e. for a 5 ml min^{-1} flow-rate, a maximum of free EGFP, whereas cell viability decreased (30%) following ultrasonic treatment. From 5 to 15 ml min^{-1} we observed a phase plateau, i.e. 30% of the cells were viable whereas fluorescence in the medium was around 70%. Beyond this value corresponding to 2.5 min residence time, the phenomenon was the contrary: i.e. more intact cells and a decrease in free EGFP.

The results obtained with the 19 mm probe were not as good; we used low fluxes to have an optimal EGFP recuperation output. This is due to the fact that the 19 mm probe occupies a large space in the

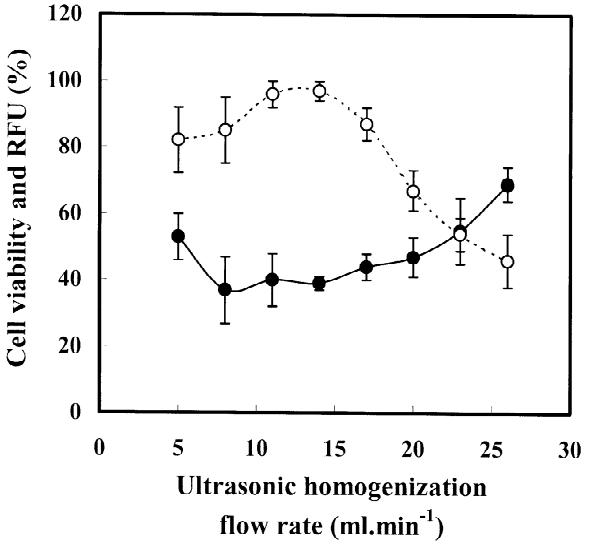

Fig. 1. The percentage of viability as well as fluorescence released by ultrasonic-treated bacteria with the 13 mm probe at various homogenization flow-rates. At the exit of the ultrasonic cell, fluorescence was determined as described in Section 2. A sample was diluted several times then a fraction of every dilution was spread onto the LB-agar-ampicillin plates. After 24 h incubation at 37 °C, the bacterial colonies were counted and compared to those obtained before ultrasonic treatment. The data represent the average of three independent experiments. ○, Fluorescence (%); ●, viability (%).

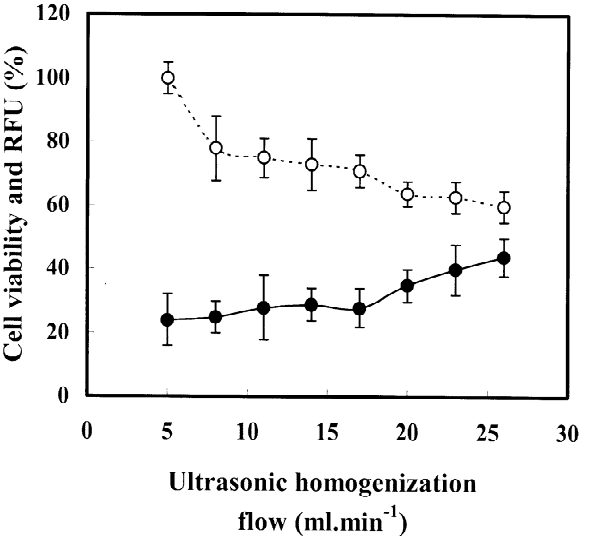

Fig. 2. The percentage of viability as well as fluorescence released by ultrasonic-treated bacteria with the 19 mm probe at various homogenization flow-rates. Samples were treated as described in Fig. 1 legend. The data represent the average of three independent experiments. ○, Fluorescence (%); ●, viability (%).

non-stop ultrasonic chamber, so the cells have a shorter duration of passage than with the 13 mm probe.

We needed large fluxes for technical reasons due to the method of IMAC purification.

3.2. Immobilized metal affinity expanded bed adsorption

Since the 13 mm probe gave the best results on the ultrasonic test, we used it during on-line process development. A 13 ml min^{-1} flow-rate was applied. The on-line purification was done with the EGFP produced in one night by bacteria in a 1.5-l bioreactor. The expansion of the bed in the column was done as described in Section 2. The coefficient of expansion was equal to 1.9 (sedimented bed=19 cm, expanded bed=36 cm).

The culture medium was cooled to 8 °C. It underwent an ultrasonic treatment at 13 ml min^{-1} flow-rate. At the exit of the non-stop ultrasonic cell, the temperature of the culture medium was 20 °C. The unclarified feedstock was applied only once and continuously through the bed on the streamline 25 column at the same flow-rate. Purification took place according to the protocol described in Section 2. A typical chromatogram is given in Fig. 3. At different stages, we took samples to follow the process of the on-line purification. The results are given in Table 1.

After the ultrasonic treatment, about 52% of the total amount of proteins released during ultrasonic treatment were fixed on the Streamline Chelating, while the rest were localized in the through-flow. The percentage of EGFP at the beginning as deduced from fluorescence represented more than 50% of the soluble proteins. After purification, the EGFP solution was eightfold concentrated (3.04 mg ml^{-1} versus 0.38 mg ml^{-1} of the homogenate). When

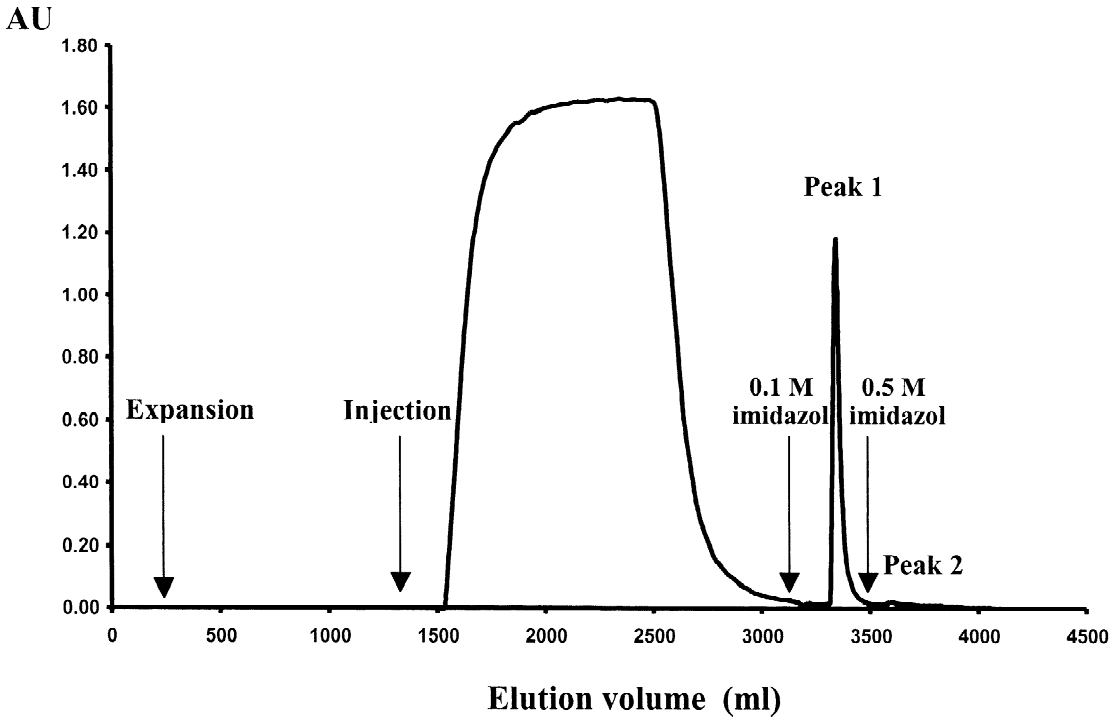

Fig. 3. Purification of EGFP by immobilized metal affinity chromatography with chelating Streamline. Column, Streamline 25 was expanded and equilibrated as described in Section 2. The application of the homogenate from the ultrasonic continuous cell was performed at 13 ml/min. After sample injection (I) and washing, the gel was sedimented and the EGFP eluted with buffer A: 0.1 *M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 8 (peak 1) and buffer B: 0.5 *M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 8 (peak 2). Detection at 280 nm; flow-rate, 780 ml h^{-1} in expansion/equilibration/feed application and in sedimented bed mode for washing/elution.

Table 1
Results of on-line purification of EGFP

	[Protein] (μg/ml)	Total protein (mg)	[EGFP] (μg/ml)	Total EGFP (mg)	EGFP recovery (%)
Starting material	748	1047	386	540	100
IMAC–N.R.	319	447	39	54	10
Peak 1	3400	544	3036	486	90
Peak 2	15	4.5	13	3.9	0.7

1.4 l of culture medium were used. After the ultrasonic treatment IMAC was performed. The non-retained fraction (N.R.) was equal to 1.4 l. The beds were sedimented, then the fixed proteins were eluted at a 180 ml h^{-1} flow-rate with a volume equal to 160 ml for peak 1 and 300 ml for peak 2.

analyzing EGFP recovery, we noticed that 90% of EGFP was in peak 1 and 0.7% in peak 2 (Fig. 3); 10% of EGFP was in the through-flow. This loss might be due to the presence of the small channels in the Streamline Chelating.

Since EGFP was very strongly induced in the bacteria in our fermentation conditions, the best factor of purification was 1.7, i.e. a higher proportion than in the other proteins in the starting homogenate.

3.3. Analysis of proteins by SDS–PAGE

Proteins (15 μg) from non-induced cell homogenate and ultrasonic-induced cell homogenate, or 8 μg of proteins obtained from the non-retained fraction or from peaks 1 and 2, were used for SDS–PAGE (Fig. 4). The analysis of the proteins contained in the different fractions of the purification showed that the EGFP His-tag had a relative molecular mass (M_r) of about 26,000. On the track corresponding to peak 1 there was a majority strip at M_r 26,000 and a minority strip at M_r 46,000. This confirms the 90% purity of the protein. The track corresponding to peak 2 showed only one strip weakly stained at M_r 26,000. Since the proteins in this peak corresponded to a low concentration (15 μg ml^{-1}), we concentrated the sample with a Vivaspin concentrator before depositing it on the electrophoresis gel. The weak stain observed on the gel could arise from a material loss during this treatment or from an overestimation of the protein quantity during the BCA measurement. 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 10% of EGFP not recovered in peak 1.

4. Conclusion

We have developed an on-line purification method including the production of a recombinant protein by *E. coli*. The cells are broken by an ultrasonic treatment and the EGFP is purified by IMAC–EBA. The whole process runs non-stop from production to purification in only one step (Fig. 5), and represents a means for industrialists to obtain a protein of interest as quickly as possible. The process is adaptable to scale-up in industry by the combination of large capacity bioreactors and ultrasonic probes at high flow-rates. A company has now developed a new 4000-W ''Pilotstation''. In the past, many ultrasonic processes were ''laboratory curiosities''

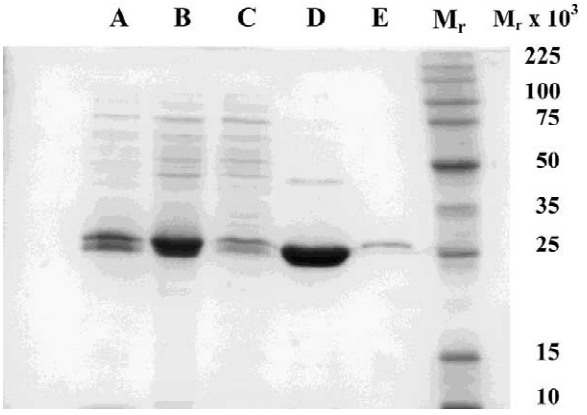

Fig. 4. Samples from the different purification steps were analyzed by SDS–PAGE electrophoresis. The proteins (15 μg) from non-induced cell homogenate and ultrasonic-induced cell homogenate or (8 μg) from non-retained fraction, peak 1 and peak 2, were loaded in each lane of the (12%) acrylamide gel. At the end of electrophoresis, the gel was Coomassie-stained. A, Non-induced homogenate; B, induced homogenate; C, non-retained fraction; D, peak 1; E, peak 2.

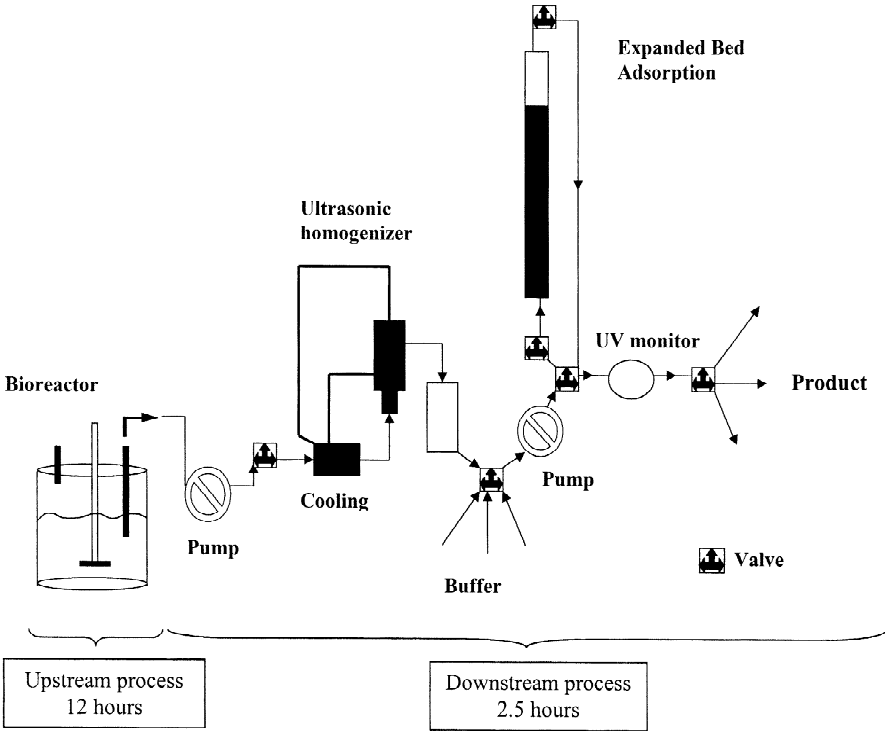

Fig. 5. Schematic representation of on-line EGFP purification process.

only, because the technology was not available to bring the output volumes up to the quantities required for commercial applications. Now, however, with the ''Pilotstation'' line of high power, large volume reactors, commercial applications in sonochemistry can become a reality, the flow-rate being about 6 l min^{-1} [20].

In our construction, EGFP is a protein possessing a histidine-tag, which is expressed under the dependence of the LacZ 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 [4], 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.

In conclusion, we obtained more than 450 mg of EGFP from 1.5 l cell culture in less than 15 h, while reducing the process of purification to three steps.

The use of EGFP allowed us to detect its presence easily throughout the experiments by fluorescence measurement. The studies of ultrasonic efficiency for the 13 mm probe led to the best flow-rate (13 ml min^{-1}) for breaking a high number of cells (64%) and releasing the corresponding EGFP (100% of fluorescence). This was verified by measuring fluorescence and the viability of the bacteria after ultrasonic treatment.

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. The ultrasonic treatment induces breaks at the level of the nucleic acids [4,21,22] that are responsible for modifying the viscosity of the culture medium, while other techniques are used to break the cells [4].

The expanded bed adsorption technology simplifies the process. The on-line ultrasonic treatment avoids wasting time so the molecule of interest can

cling very quickly to the IMAC support. We therefore conclude that on-line bioreactor–ultrasonic probe–immobilized metal affinity expanded bed adsorption is a rapid automated technique for obtaining large quantities of pure EGFP.

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