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Evaluation of three expanded bed adsorption anion exchange matrices with the aid of recombinant enhanced green fluorescent protein overexpressed in *Escherichia coli*

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Abstract

Three anion exchanger expanded bed adsorption (EBA) matrices: Streamline DEAE, Streamline Q XL and Q Hyper Z were evaluated with the aid of EFGP from an ultrasonic homogenate of *Escherichia coli*. Two pH of buffer were tested. Capture was done in an expanded mode whereas elution was done in a packed mode. The same conditions were chosen for evaluation of the three matrices. We observed a loss of EGFP (8–15%) in the through flow fraction especially with the Streamline Q XL matrix, probably due to an aggregation of beads during sample application. The beads of this matrix possess tentacles which probably retain a lot of cellular and molecular debris. The two other matrices gave a good purification of the EGFP (7–15-fold) but the Q Hyper Z matrix appeared to give the best results. It is composed of little size and density beads which lead to a higher exchange surface and then a better mass transfer.

Keywords: *Escherichia coli*; Expanded-bed adsorption anion exchange matrices; Enhanced green fluorescent protein

1. Introduction

An alternative to traditional clarification (centrifugation, tangential micro and ultrafiltration and the first chromatography step), expanded bed adsorption (EBA) may be used to process directly large volumes of crude feedstock [1–3]. 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]. 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 significant. Increase in 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 of nucleic acids that are responsible for modifying the viscosity of the culture medium when other techniques are used to break the cells [5–7]. Thömmes proposed a number of design criteria to improve separation performance in EBA [8]. These included a reduction in particle size to reduce the length for protein diffusion, in conjunction with an increase in density to allow the use of high flow-rates, and a gradient in support size and/or density to limit axial dispersion. Karau et al. [9] showed that reducing the diameter of a DEAE-linked adsorbent led to improved breakthrough capacities for BSA and that mass transfer within the liquid film surrounding the support then became one of the limiting factors controlling protein adsorption. In addition to the mass transport benefits of small supports, the axial dispersion in expanded beds of a given voidage is generally lower for smaller particles [10,11].

The present paper describes the evaluation of three expanded bed adsorption anion exchanger matrices: Streamline DEAE, Streamline Q XL and Q Hyper Z, a new adsorbent of the market. The two Streamline matrices have the same density but the Streamline Q XL possesses tentacles and is given by the manufacturer to have a higher capacity than Streamline DEAE matrix. The Q hyper Z is composed of high density beads and given by the manufacturer to

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Table 1
Properties of the three expanded bed adsorption anion exchanger matrices

	Streamline Q XL (Amersham Biosciences)	Streamline DEAE (Amersham Biosciences)	Q HYPERZ (BioSepra)
Ionic capacity	0.23–0.33 mmol Cl^- ml^{-1} adsorbent	0.13–0.21 mmol Cl^- ml^{-1} adsorbent	0.15–0.20 mmol Cl^- ml^{-1} adsorbent
Matrix structure	Macroporous cross-linked 6% agarose with crystalline quartz core material and bound dextran	Macroporous cross-linked 6% agarose with crystalline quartz core material	Zirconium oxide
Particle form	Spherical, 100–300 μm	Spherical, 100–300 μm	Spherical, 45–105 μm
Mean particle size	200 μm	200 μm	75 μm
Mean particle density	1.2 g ml^{-1}	1.2 g ml^{-1}	3.2 g ml^{-1}
Recommended working flow-rate	300–500 cm h^{-1}	300–500 cm h^{-1}	250–500 cm h^{-1}
Binding capacity	>110 mg BSA ml^{-1} adsorbent	>40 mg BSA ml^{-1} adsorbent	>40 mg BSA ml^{-1} adsorbent

have the same capacity as the Streamline DEAE. Properties of the three matrices are summarized in Table 1.

Evaluation of the three matrices was performed with crude extract of enhanced green fluorescent protein (EGFP) overexpressed in *Escherichia coli*. Since the cloning of its gene which started from the jellyfish *Aequorea victoria* [12], the green fluorescent protein (GFP) has been widely used in cellular biology [13,14]. 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 [15]. We used the EGFP as an easily quantifiable protein by fluorometric assay to evaluate the best matrix for the EGFP purification.

2. Experimental

2.1. Instruments

The chromatographic system used throughout this study was the Biopilot workstation from Amersham Biosciences (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 electrophoresis apparatus used was the Mini-Protean II from BioRad (Marnes-la-Coquette, France).

2.2. Chemicals

Streamline DEAE matrix, Streamline Q XL matrix and Streamline 25 column were from Amersham Biosciences (Saclay, France). Q Hyper Z matrix was from BioSepra (Cergy-Saint Christophe, 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. Vivaspin concentrators were from Sartorius (Palaiseau, France).

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. Cloning was done as previously described [16]. The PCR product was inserted into the pET 15b plasmid to create the EGFP construction which 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 mM 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 about 400 ml of cell culture was performed at 20 kHz with a 19 mm probe. Four 1 min pulses with 1 min in ice between each pulse were performed. A dilution step was performed for the sample. Then, pH and conductivity were controlled and adjusted if necessary.

2.6. Expanded bed adsorption

2.6.1. Measuring the degree of expansion

The degree of expansion is calculated from the ratio of expanded bed height (L) to sedimented bed height (L_0). A significant decrease in the degree of expansion may indicate poor stability or channelling due to trapped air under distributor plate, infection or fouling of the adsorbent, the column not being in vertical position, or a blocked distributor plate. Therefore, absolute values for the degree of expansion can only be compared if the buffer system (liquid density and viscosity) and temperature are constant between runs. To study the expansion versus flow-rate, increments of 0.5 ml min^{-1} of the flow-rate were used from 0.5 ml min^{-1} until the flow-rate necessary to obtain a three-fold degree of expansion.

2.6.2. Anion exchange expanded bed adsorption

The experiments were performed with Streamline DEAE, Streamline Q XL and Q hyper Z (100 ml, 20 cm sedimented gel) packed in the Streamline 25 column according to the manufacturer's procedure. The column was linked to a Biopilot workstation. Equilibration/expansion was performed with Tris–HCl 50 mM pH 8 or 9 buffer with the flow-rate previously determined to obtain the sufficient degree of expansion. The unclarified feedstock was then applied at this flow-rate, 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 column volumes (sedimented gel) of equilibration buffer, elution was performed with linear gradient during 20 column volumes from 0 to 40% and then to 100% with NaCl 1 M, Tris–HCl 50 mM pH 8 or 9 buffer using a downward flow.

2.7. Analytical procedures

2.7.1. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [17] 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.

2.7.2. 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 of Relative Fluorescence Units versus quantity of pure EGFP was used to determine the quantity of EGFP in samples, i.e. free by ultrasonic treatment and then purified.

2.7.3. Protein concentration

The protein concentration was estimated by Bicinchoninic acid assay [18] using bovine serum albumin as standard.

3. Results and discussion

3.1. Degree of expansion

We observed the expansion behaviour of the three matrices. The bed expansion degree, L/L_0, is shown in function of liquid flow-rate (Fig. 1). The three matrices showed a linear fluidization when increasing the flow-rate and the bed expansion is stable and homogenous. For the two Streamline matrices, an expansion degree of 3 is obtained with a flow-rate of 30 ml min^{-1}. For the Q Hyper Z matrix, an expansion degree of 3 is obtained with a flow-rate of 51 ml min^{-1}.

To obtain a stable bed expansion, all the beads must experience a flow around them equal to their individual terminal settling velocities u_p. A particle can be assumed to be axially stationary when its settling velocity in the bed is equal and opposite of the upward liquid interstitial velocity at that point in the bed. At the stationary state of the particle, $u_t = u_p$. Then, to determine the terminal fluidization velocity u_t, we can use the particle terminal velocity u_p in the fluid which is described by:

$$u_t = u_p = \frac{g d_p^2 (\rho - \rho_L)}{10\eta(1 + 0.15\, Re_p^{0.687})} \quad (1)$$

This law is more applied than Stokes' law for an EBA medium where interactions between particles are not negligible. The Stokes' law corresponds to a case of weakly concentrated suspensions [19]. Nevertheless, for more highly concentrated suspensions, it is suitable only if the particle Reynolds number is low ($Re_p < 0.2$).

When there is more than one particle, because of interaction between the beads, inspection of these laws shows that the terminal fluidization velocity is dependent on (i) the difference in density between bead and fluid, (ii) the viscosity of the fluid, (iii) the section of column and (iv) the bead diameter. Of all of them, bead diameter, squared in the Eq. (1) is the most important parameter.

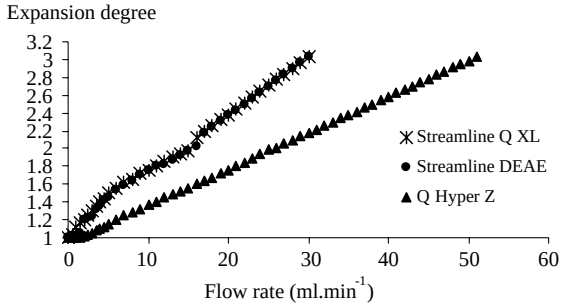

Fig. 1. Bed expansion degree in function of increasing flow-rate for the three expanded bed adsorption anion exchanger matrices.

So, the two Streamline matrices had the same comportment since their beads have the same diameter and density. Obviously, the tentacles of the Streamline Q XL matrix did not modify the fluidization of the gel. The Q Hyper Z needed higher flow-rate to reach the same expansion degree since its beads have a littleness diameter and density.

3.2. Evaluation of the three expanded bed adsorption anion exchanger matrices with EGFP fro ultrasonic homogenate of E. coli

After the cell disintegration step with ultrasonic homogenisation, the culture medium named as crude extract (with pH adjusted to 8 or 9) was loaded directly onto the expanded bed adsorption column. The fluid velocity previously determined was maintained during all the run. The surface of the expanded bed could be easily observed even in the presence of turbid particulate materials. We observed that the expanded bed remained stable when unclarified feedstock was applied to the adsorbent bed and that there was no back mixing and liquid channels present in the column, except for the Streamline Q XL matrix. We observed too an increase of bed height during feed loading for the Q Hyper Z matrix and a decrease for the two Streamline matrices. After washing of the expanded bed, adsorbed proteins were eluted from the adsorbent in a packed bed mode to reduce dilution of the product in the eluate. The elution step was used at a constant flow-rate with downward flow. The obtained chromatograms are given in Figs. 2 and 3. It is advisable to specify that the comparison of the three matrices was carried out under precise conditions that were selected to evaluate the supports but that were not optimized for the purification of the EGFP. This will be the subject of a later study. At pH 8, for the three matrices, we observed two peaks more or less distinct followed by a shoulder (Fig. 2). The EGFP was detected in the second peak. At pH 9, we also obtain two peaks followed by a shoulder where we detected the EGFP (Fig. 3). At different stages, we took

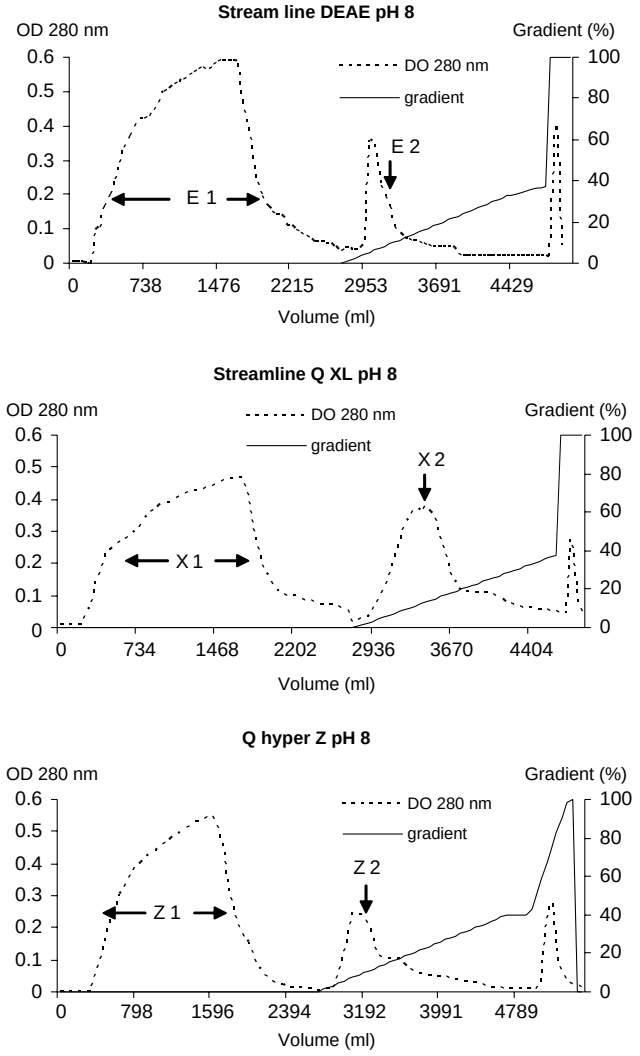

Fig. 2. Purification of EGFP by expanded bed adsorption with the Streamline DEAE, the Streamline Q XL and the Q hyper Z matrices at pH 8.

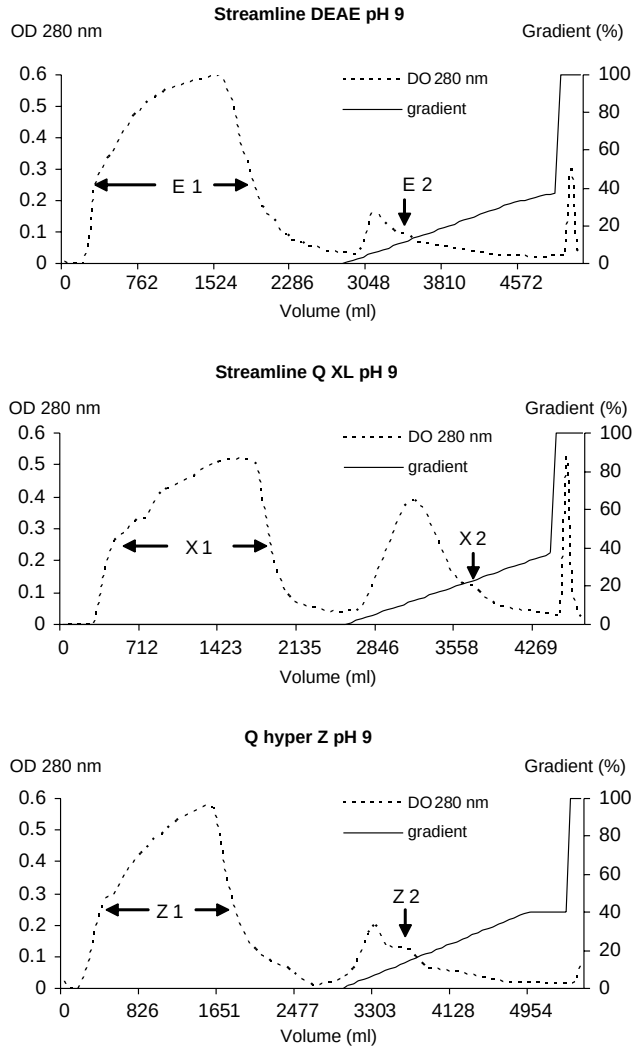

Fig. 3. Purification of EGFP by expanded bed adsorption with the Streamline DEAE, the Streamline Q XL and the Q hyper Z matrices at pH 9.

Table 2
Results of purification of EGFP with the three evaluated matrices at pH 8 and 9

		Volume (ml)	Protein (mg ml^{-1})	Total protein (mg)	Total EGFP (mg)	Specific fluorescence (mg EGFP mg^{-1} total protein)	Yield (%)	Purification
Streamline DEAE (pH 8)	Crude extract	386	3.4	1312.4	83.4	0.06		1
	Through flow	2100	0.39	819.0	8.9	0.01	11	0
	Eluate	150	0.89	133.5	56.0	0.42	67	7
Q Hyper Z (pH 8)	Crude extract	386	3.4	1312.4	83.4	0.06		1
	Through flow	2000	0.41	820.0	6.5	0.01	8	0
	Eluate	300	0.27	81.0	72.3	0.89	87	15
Streamline Q XL (pH 8)	Crude extract	380	3.4	1292.0	83.4	0.06		1
	Through flow	1900	0.4	760.0	12.6	0.02	15	0
	Eluate	400	0.17	68.0	38.9	0.57	47	10
Streamline DEAE (pH 9)	Crude extract	390	3.4	1326.0	84.0	0.06		1
	Through flow	2200	0.39	858.0	9.6	0.01	11	0
	Eluate	300	0.32	96.0	68.9	0.72	76	12
Q Hyper Z (pH 9)	Crude extract	390	3.4	1326.0	84.0	0.06		1
	Through flow	1900	0.42	798.0	9.6	0.01	11	0
	Eluate	300	0.3	90.0	68.9	0.77	78	13
Streamline Q XL (pH 9)	Crude extract	390	3.4	1326.0	84.0	0.06		1
	Through flow	2000	0.41	820.0	13.2	0.02	15	0
	Eluate	200	0.42	84.0	34.2	0.41	39	7

samples to follow the process of the purification. The results are given in the Table 2. In all cases, we observed a loss of EGFP in the through flow fraction especially with the Streamline Q XL matrix. Yield results showed the presence of 8–15% of EGFP in the through flow fractions. These gels are very capacitive; also we supposed the formation of preferential channels on the level of the gel. However this was observed only in the case of the Streamline Q XL matrix. In spite of that, we found a correct degree of purification for the three matrices between 7 and 15. The yield results are relatively good in particular for the Q Hyper Z matrix (87% at pH 8). The major difference between the two Streamline matrices and the Q Hyper Z matrix is the beads size and density. We have used the same volume of each matrix. We have determined the outer exchange surface of each matrix in the volume V_c:

$$S_T = \frac{3\phi V_c}{r} \quad (2)$$

This equation is done by the apparent surface of a bead $S_{APP} = 4\Pi r^2$ and by the solid voluminal fraction of the bed $\phi = V_b/V_c$. It appeared that the total exchange surface of the Q Hyper Z matrix (4.84 m^2) was three-fold greater than that of the DEAE Streamline matrix (1.81 m^2). Moreover due to the very resistant mineral skeleton of Q Hyper Z beads, they are filled with a high charge-density cross linked hydrogel. This could explain the best yield and purification observed with the Q hyper Z matrix. Nevertheless, the Streamline Q XL matrix possesses tentacles which might give it a higher total exchange surface. It would seem that it's really the case but that a lot of particulates are retained by the beads. Indeed, we observed a lot of aggregates during the sample loading and consequently an inhomogeneous expansion. The composition of feedstocks has been extremely well described by Anspach et al. [20]. These authors described the major components of feedstock, i.e. particulates (cells, cell fragments, protein precipitates) and dissolved constituents of low and high molecular mass. They found that feedstocks were more viscous than equilibration buffers, especially bacterial feedstocks [20]. Other authors found that during application of feedstock, the bed height increases even if the flow-rate is kept stable, owing to the higher viscosity of the feedstock [21]. A moderate increase does not affect the stability of the bed. Samples with very high viscosity, which causes significant increases, can cause channelling in the column with a subsequent early breakthrough of the protein of interest [20,22]. This phenomenon was increased with the presence of tentacles on the beads of the Streamline Q XL matrix. The high viscosity feedstocks are essentially due to the presence of folded and unfolded long chain DNA released during the growth phase or during cell lysis for protein recuperation, in spite of the ultrasonic treatment. A DNase I treatment and/or a sample dilution can significantly reduce the viscosity [20,23]. A homogenate of *E. coli* was used as sample in order to study the efficiency of different CIP procedures. In addition to the expressed protein, EGFP, this suspension also contains ~2000–3000 different proteins, as well as compounds such as lipids, fatty acids, nucleic acids and amino acids. When a nuclease (Benzonase) was added to the *E. coli* suspension this allowed avoiding aggregation of Streamline Q XL caused by DNA. Without benzonase, aggregation of Streamline Q XL was observed. The effect of Benzonase on adsorption to Streamline Q XL may be attributed to the fact that small oligonucleotides cannot sterically hinder adsorption of amino acids or proteins [23].

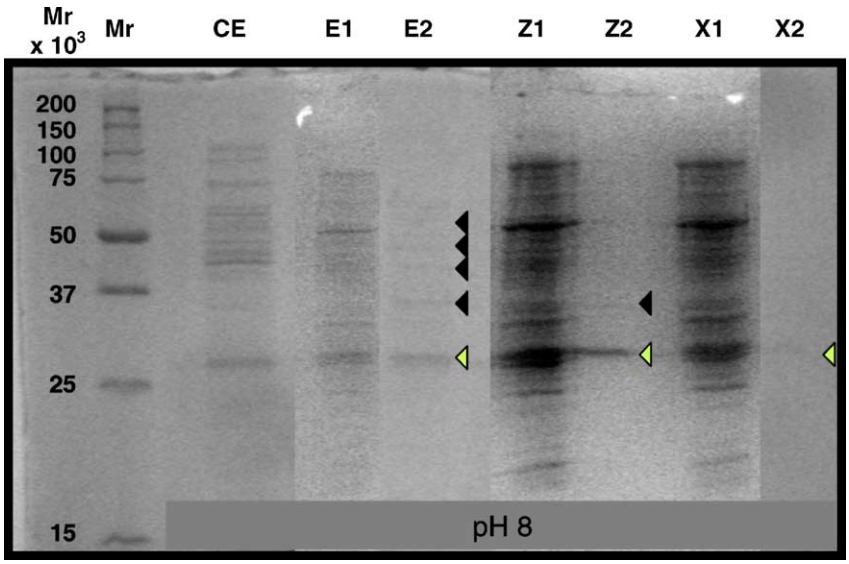

Fig. 4. Analysis of samples from expanded bed adsorption at pH 8 by SDS-PAGE. Mr: molecular mass markers; CE: initial crude extract; E1: flow through fraction from Streamline DEAE matrix (21*x* concentrated); E2: EGFP eluted fraction from Streamline DEAE matrix; Z1: flow through fraction from Q Hyper Z matrix (75*x* concentrated); Z2: EGFP eluted fraction from Q Hyper Z matrix; X1: flow through fraction from Streamline Q XL matrix (75*x* concentrated); X2: EGFP eluted fraction from Streamline Q XL matrix.

The sample must be treated before loading on the Streamline Q XL matrix. For example, desoxyribonuclease and $MgCl_2$ can be used to decrease viscosity and improve the expansion and the purification.

3.3. Analysis of proteins by SDS-PAGE

Proteins from crude extract and proteins from through flow fraction and EGFP fraction were analysed by SDS-PAGE (Figs. 4 and 5). The analysis of the proteins of EGFP fractions showed that the EGFP had a relative molecular mass (Mr) about 30,000. Since the through flow fractions corresponded to a low protein concentration, we concentrated the samples with a Vivaspin concentrator before deposing it on the electrophoresis gel. The observed loss of EGFP in the through flow fraction was confirmed by electrophoresis for the two tested pH. Moreover, the electrophoresis showed that the fractions containing the EGFP were not pure. This was not surprising according to the chromatograms. With the Streamline DEAE matrix, a

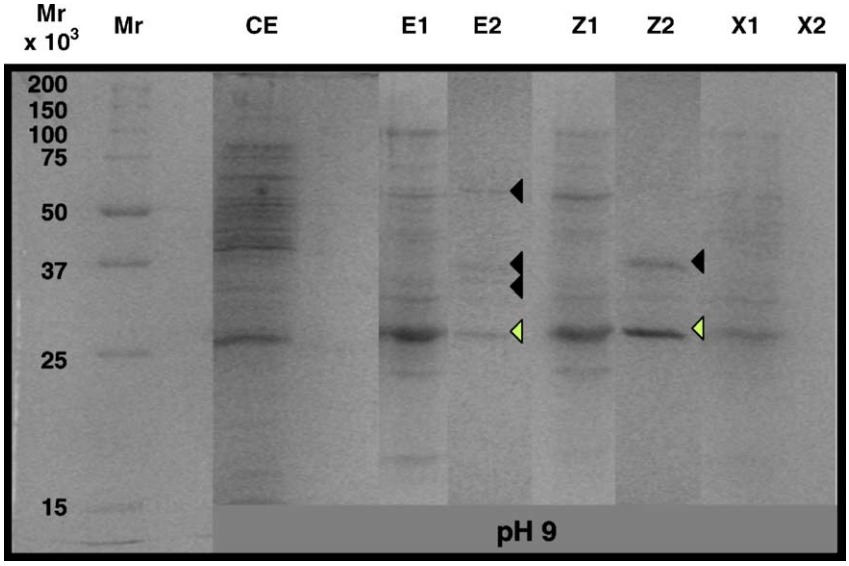

Fig. 5. Analysis of samples from expanded bed adsorption at pH 9 by SDS-PAGE. Mr: molecular mass markers; CE: initial crude extract; E1: flow through fraction from Streamline DEAE matrix (30*x* concentrated); E2: EGFP eluted fraction from Streamline DEAE matrix; Z1: flow through fraction from Q Hyper Z matrix (50*x* concentrated); Z2: EGFP eluted fraction from Q Hyper Z matrix; X1: flow through fraction from Streamline Q XL matrix (30*x* concentrated); X2: EGFP eluted fraction from Streamline Q XL matrix.

lot of weak bands were found with the EGFP fraction (E2) while with the Q Hyper Z matrix only one contaminant protein was found (Z2). This result was already observed in on-line purification of EGFP with immobilized metal affinity expanded bed adsorption [24].

4. Conclusion

In this paper, we show that the three matrices are suitable for capture of EGFP but with different degree of recovery of the protein. Q HYPER Z beads have been developed based on the optimal combination of bead diameter and density. As a result, they can be used in expanded bed operations at high linear flow-rates without compromise to their high protein binding capacities, in contrast to conventional agarose beads found which constitute the two Streamline matrices. The high density of Q HYPERZ beads also allows for efficient processing of feedstocks that are very high in biomass and/or viscosity in contrast to the tentacles of the Streamline Q XL. In spite of this, we think that optimisation of EGFP purification condition could lead for the three matrices to obtaining a pure EGFP (pH buffer, elution, ...). Nevertheless, it will probably need precondition treatments of the sample with the Streamline Q XL matrix. It is probably more adapted to secreted proteins. This would be the aim of a later study.

5. Nomenclature

d_p	particle diameter (m)
g	acceleration due to gravity ($m\,s^{-2}$)
L	expanded bed height (m)
L_0	settled bed height (m)
r	radius of beads (m)
Re_p	particle Reynolds number
S_T	total exchange surface of a given volume of matrix (m^2)
u_p	bead terminal settling velocity ($m\,s^{-1}$)
u_t	terminal fluidization velocity ($m\,s^{-1}$)
V_b	beads volume (m^3)
V_c	column volume (m^3)

Greek letters

η	suspension (beads and fluid) viscosity (Pa s)
ρ_L	fluid density ($kg\,m^{-3}$)
ρ	bead density ($kg\,m^{-3}$)
ϕ	solid voluminal fraction of the bed

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