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Endostatin capture from *Pichia pastoris* culture in a fluidized bed From on-chip process optimization to application

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Abstract

One of the characteristics of the methylothrophic yeast *Pichia pastoris* is its ability to grow to a very high cell density. Biomass concentrations of 300–400 g wet mass/l are common. It is therefore obvious that the recovery processes of extracellular proteins from this microorganism should take into account the effect of high biomass content. Separation by filtration and/or centrifugation is possible but these steps are cumbersome and can affect the protein recovery. The use of fluidized beds is attractive proteins capture option since it eliminates the biomass while capturing the desired protein. Zirconia-based resins possess unique properties which make them appropriate for processing high biomass concentrations in an expanded bed mode. The beads are particularly heavy (density is 3.2 g/ml) and small (75 μm) and therefore can accommodate high fluidization velocity and high mass transport. Specific operating conditions for effective capture of expressed protein have to be determined. This determination is generally time consuming and requires relatively large amount of feedstock for the lab trials. To avoid multiple chromatographic trials in columns, optimal conditions of adsorption and elution were determined by ProteinChip technology coupled with mass spectrometry. This technology involves flat chip surfaces functionalized as chromatographic beads where it is possible to adsorb and desorb proteins. Four different functional groups (strong anion-exchange, weak cation-exchange, hydrophobic and metal chelate) were tested and the retained proteins were analyzed directly by mass spectrometry. The weak cation-exchange group was chosen for further work. The Zirconia-based weak cation-exchange sorbent (CM HyperZ) was evaluated for binding capacity in a packed column and then for capturing endostatin from crude feed stock. Based on the previously determined conditions; 45 l of culture containing approximately 15 kg of biomass (wet mass) and 3 g endostatin were applied on an expanded bed at a flow-rate of 535 cm/h, yielding 80% of the endostatin and removing approximately 80% of foreign proteins.
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1. Introduction

Endostatin, a M_r 20 000 fragment of collagen XVII with an isoelectric point of 9.1 [1], is a compound with potential anti cancer activity. Initial studies indicated that this molecule can inhibit blood vessel formation and consequently inhibit tumor growth [2,3]. In its recoimbinant form, this protein is currently expressed in the metylotrophic yeast *Pichia pastoris*. This microorganism is an efficient producer of recombinant eukaryotic proteins [4,5], it grows to high biomass concentrations, 300–400 g wet cells/l are common [6], and in most cases it secretes the

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produced protein to the growth media. High biomass concentration is a major obstacle in protein recovery processes, especially when the proteins are secreted. Removing large amounts of biomass is difficult, cumbersome and can lead to poor yield.

To overcome the risk of reducing protein recovery due to removal of large biomass, the fluidized bed adsorption process appears as an efficient approach. Here, the expressed protein (in this case endostatin) is captured directly from the unclarified feed stock on solid-phase adsorbent while particles in suspension, such as cells and cell debris are eliminated [7–9]. The solid-phase adsorbent plays an important role since its fluidization is dependent on particle size and bead density. It is generally recognized that particle size should not be large because it would increase the mass transfer resistance and therefore reduce the dynamic binding capacity. Particle density would have to be high to keep high flow-rates while maintaining the expansion factor within reasonable ranges [10–12].

Despite the fact that technologically a fluidized bed separation would present advantages over packed bed when high biomass is an issue, it is important to make the appropriate choice in terms of sorbent functionality, and to optimise the adsorption and elution phases for the target protein. Protein separation process design is a relatively long procedure built essentially on a trial and error approach. Although the isoelectric point of the target protein or biological properties are frequently considered for initial choice of sorbent and solvent conditions, the standard approach is to make separation mappings on most common resins. Using this approach, it may be possible to identify the appropriate conditions that allow specific capturing of the target protein. Chromatographic fractions are collected and analyzed by classical electrophoresis or high-performance liquid chromatography (HPLC) methods to assess capture efficiency and purity. Recently, it has been demonstrated that the application of mass spectrometry strategies associated with ion-exchange surfaces is an efficient and rapid way to develop a process chromatography protocol [13]. When implementing this approach, it is important that identical chemistry will be available both on the solid-phase sorbent and the mass spectrometry surface, so the same interaction with the proteins occurs. This approach saves a significant amount of time and labor and is directly usable in a column.

This paper describes the specific capture of recombinant endostatin from fermentation broths by fluidized bed mode, where the separation conditions are defined by mass spectrometry.

2. Experimental

2.1. Chemicals, biologicals and equipment

ProteinChip arrays of strong anion-exchange (SAX2), weak cation-exchange (WCX2), hydrophobic interaction (H4), immobilized metal affinity capture (IMAC3), and normal-phase (NP 20) were tested. These arrays were from Ciphergen Biosystems (Fremont, CA, USA). The processed arrays were read using a laser desorption ionization time-of-flight mass spectrometer, PBS II reader (SELDI) from Ciphergen Biosystems CM HyperZ beads, carboxymethylated sorbent for a cation-exchange fluidized bed based on zirconium oxide porous beads, were from the Ciphergen Bioprocess Division (Cergy, France). Fluidized bed columns were from UpFront Chromatography (Denmark), and from Amersham Biosciences (Piscataway, NJ, USA). An 80 l Bioflo 5000 fermentor was from New Brunswick Scientific (NJ, USA). A phenylmethylsiloxane column (30 m×250 μm I.D., 2.05 μm film thickness) was purchased from Hewlett-Packard, USA. HiTrap SP HP was from Amersham Biosciences (Piscataway, NJ, USA). A BCA protein quantification kit was supplied by Pierce (Rockford, IL, USA). Pre-casted polyacrylamide gels for electrophoresis came from BioRad Labs., Ivry sur Seine, France. Simply Blue SafeStain for gel staining was purchased from Invitrogen (CA, USA). All chemicals used all along the study were from Aldrich, Brussels, Belgium and were of analytical grade.

2.2. Pichia pastoris cell culture

P. pastoris strain GS 115His$^+$ Mut$^+$ expressing mouse endostatin was prepared as described earlier [14]. Pilot scale fermentation was performed in 80 l Bioflo 5000 fermentor. The fermentor was interfaced and controlled via an adaptive control algorithm [15]

that maintained dissolved oxygen level at 30% saturation by adjusting the agitation rate and the supply of air. Initially 25 l BMGY culture medium (peptone 20 g/l, yeast extract 10 g/l, glycerol 30 g/l, yeast nitrogen base 13.4 g/l, potassium phosphate monobasic 11.9 g/l, potassium phosphate dibasic 2.14 g/l, biotin 0.0001 g/l) were inoculated with 1.0 l overnight-culture and grown in batch mode for approximately 16 h (A_{600}~80). At this stage, a fed-batch mode started by adding a 50% glycerol solution containing 12 ml/l trace element solution at a flow-rate of 18 ml h^{-1}. After another hour (optical density A_{600}~100), the glycerol feed rate was reduced to 2 ml l^{-1} h^{-1} and addition of methanol containing 12 ml l^{-1} trace element solution commenced at an exponential pump rate controlling the yeast growth rate at 0.02 h^{-1}.

Off-line methanol measurements were done using gas chromatography coupled to a mass-selective detector, and 1 μl sample was analyzed in a 5% phenylmethylsiloxane column. The column was programmed to raise 5 °C/min from 50 to 150 °C. The flow-rate of the helium carrier gas through the column was 1.2 ml/min. The temperature of the injector was 200 °C.

2.3. Endostatin determination

Samples taken during the course of induction were centrifuged at 4000 *g* for 40 min; the supernatant was diluted to adjust the conductivity to 5.5 mS/cm and the pH to 6.0. The samples were then loaded on 5 ml HiTrapSP HP at a ratio of 20 mg total protein per ml packed resin, and the column was washed with 10 column volumes of 20 m*M* phosphate buffer pH 6.0 containing 50 m*M* sodium chloride. The endostatin was eluted with 3 column volumes of 20 m*M* phosphate containing 1.5 *M* sodium chloride. The eluted samples were loaded on 4–20% precasted sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) under reducing condition. After staining with Simply Blue SafeStain the amount of endostatin was determined by digitizing and calculating using NIH image analysis software.

2.4. Selection of interactive surface array and optimization of capture conditions

Pichia pastoris cell culture supernatant was directly deposited on ProteinChip array surfaces according to a previously described method [13]. Four types of arrays were selected: cationic with quaternary amino groups (SAX2), anionic with carboxyl groups (WCX2), hydrophobic with aliphatic chains (H4) and metal chelating acid groups (IMAC3). Different physico-chemical interaction conditions were investigated (see Table 1). Each single spot was equilibrated three times with 200 μl of the investigated buffer for 5 min. Then each spot surface was loaded with 50 μl of the sample previously adjusted to the corresponding pH and ionic strength. After an incubation period of 30–60 min under shaking, each spot was then washed three times with 200 μl with the appropriate buffer to eliminate non-adsorbed proteins, followed by a quick rinse with deionized water.

All surfaces were dried and loaded twice with 0.8 μl of energy adsorbing molecule solution composed of a saturated sinapinic acid in 50% acetonitrile containing 0.5% trifluoroacetic acid and dried again. All arrays were then analyzed using a mass spectrometer reader used in a positive ion mode, with an ion acceleration potential of 20 kV and a detector

Table 1
Experimental conditions for the study conducted with chip surfaces

Spot surface	pH range	NaCl (or imidazole) conc. range (m*M*)	Type of buffer (m*M*)
WCX2 (cation-exchange surface)	4.5–6.0	0–1000	50 acetate or citrate
SAX2 (anion-exchange surface)	7.5–9.0	0–1000	50 Tris–HCl
H4 (hydrophobic surface)	7.5	1500–0	50 Tris–HCl
IMAC4 (chelating surface)	7.0	0–500 imidazole 0–500 NaCl	20 phosphate

IMAC surface was first loaded with copper or nickel ions.

gain voltage of 2 kV. The molecular weight range investigated by mass spectrometer was from 3000 to 200 000, laser intensity responsible for the desorption of protein on the spot surface was set between 200 and 280 units depending on the sample tested.

Experiments were performed in buffers of different ionic strength and pH in order to identify the array where the endostatin adsorbs easily, and then desorption conditions were identified. In the case of ion-exchange surfaces (e.g. WCX2 and SAX2), the influence of pH and ionic strength was investigated. Hydrophobic H4 spot surface was used at neutral pH over a wide range of ionic strength. IMAC3 spot surfaces, previously loaded with copper or nickel ions (copper sulfate or bickel sulfate at 100 m*M* in water) were evaluated at neutral pH (PBS pH 7.4) and in the presence of different concentrations of sodium chloride and imidazole.

2.5. Capture of endostatin in packed and fluidized bed columns and determination of binding capacity

CM HyperZ is a weak cation-exchanger made of Zirconia porous beads filled with a carboxyl-containing hydrogel. This sorbent is designed for fluidized bed applications in protein separations. Its density is about 3.2 g/ml with an average particle size of 75 μm (see Ref. [16] for detailed characterization of the sorbent).

The capture and assessment of dynamic binding capacity of endostatin from *Pichia pastoris* culture supernatant were conducted in both packed and fluidized bed conditions to evaluate possible differences. Both experiments were performed using CM HyperZ resin chosen for further fluidized bed experiments. For packed bed experiments, columns of 0.3×10 cm I.D. were first equilibrated with an adsorption buffer of ionic strength and pH determined by the preliminary experiments using array systems (i.e. 50 m*M* acetate buffer pH 5.0, see above). The pH and conductivity of 800 ml of centrifuged broth were adjusted to 5.0 and 10 mS/cm, respectively, and directly loaded onto the column. Elution was performed according to the information obtained from the preliminary mass spectrometry experiments, using two sodium chloride concentration steps. Finally, the sorbent was regenerated by a wash with 5 column volumes of 1 *M* sodium hydroxide. Chromatography separations were accomplished at a linear flow-rate of 300 cm/h. Breakthrough of endostatin was monitored by mass spectrometry and by electrophoresis.

For fluidized bed experiments, 7.8 ml of settled resin were introduced into a 1 cm diameter Fastline fluidized bed column. Solutions were always introduced from the bottom of the column for all phases of separation: equilibration, loading, washing and elution. Equilibration buffer was 50 m*M* acetate, pH 5.0. Under a linear flow-rate of 350 cm/h, the column expansion factor was about 2. A 6000-ml volume of crude feed stock (adjusted to pH 5 and diluted to give a final conductivity of 10 mS/cm), containing about 30 μg/ml of endostatin, was introduced continuously at the same flow-rate. The column was then washed with the equilibration buffer to eliminate non-adsorbed components. Elution was performed by running a two-step process: the first using a 200 m*M* sodium chloride, the second using 800 m*M* sodium chloride concentration. These conditions were extrapolated from data obtained from previous array trials. Collected fractions were analyzed by mass spectrometry and by SDS–PAGE.

2.6. Fluidized bed endostatin capture with unclarified feed stock

A sample containing 1500 ml of settled CM HyperZ was introduced into a 10 cm internal diameter fluidized bed column. The column was equilibrated with 50 m*M* acetate buffer, pH 6.0. Under a linear flow-rate of 535 cm/h the column expansion factor was 3. Then 43 liters of crude feed stock containing approximately 70 μg/ml endostatin, (adjusted to pH 6.0 and diluted with water to 110 l to get a final conductivity of 10 mS/cm) were introduced continuously at the same flow-rate. The column was then washed with the equilibration buffer to remove non-adsorbed molecules. Elution was achieved in packed bed mode by increasing sodium chloride concentration. Collected fractions were analyzed by SDS–PAGE, and protein concentration was assayed using a BCA protein assay kit (Pierce).

2.7. SDS–PAGE polyacrylamide gel electrophoresis

Electrophoresis of chromatography fractions was performed in classical conditions using 15-well pre-

casted 18% polyacrylamide gel plates. Samples were prepared by a 2-fold dilution in Laemli sample buffer. Then, 12 μl samples were loaded per lane, and electrophoretic migration was performed using a tension of 200 V for 45 min. Staining was achieved using Coomasssie blue solution in ethanol and acetic acid for 1–1.5 h under gentle agitation. Destaining was performed using 40% ethanol, and 10% acetic acid in water.

3. Results and discussion

3.1. Binding and elution of endostatin from Pichia pastoris supernatant on ProteinChip array

Initial analysis of endostatin adsorption to the four different binding groups on the arrays shown in Fig. 1. Total protein concentration in the feedstock was about 3.5 mg/ml, and the endostatin concentration was about 60 μg/ml. The cation-exchange array, WCX2, adsorbed endostatin along with minor impurities, while all other array surfaces tested did not show any adsorption of endostatin. The arrays with hydrophobic surface and metal chelate adsorbed two major impurities of M_r 25 000 and 17 000. Based on these mass spectrometry results, it is very likely that carboxymethyl resins will be the most appropriate approach for preparative purification. According to pH and ionic strength mapping studies, the optimal pH for adsorption on WCX2 is around 5.0 (50 m*M* acetate buffer). Desorption studies were done using increasing salt concentration in the loading buffer (Fig. 2). The desorption occurred when the sodium chloride concentration in the buffer was below 300 m*M*.

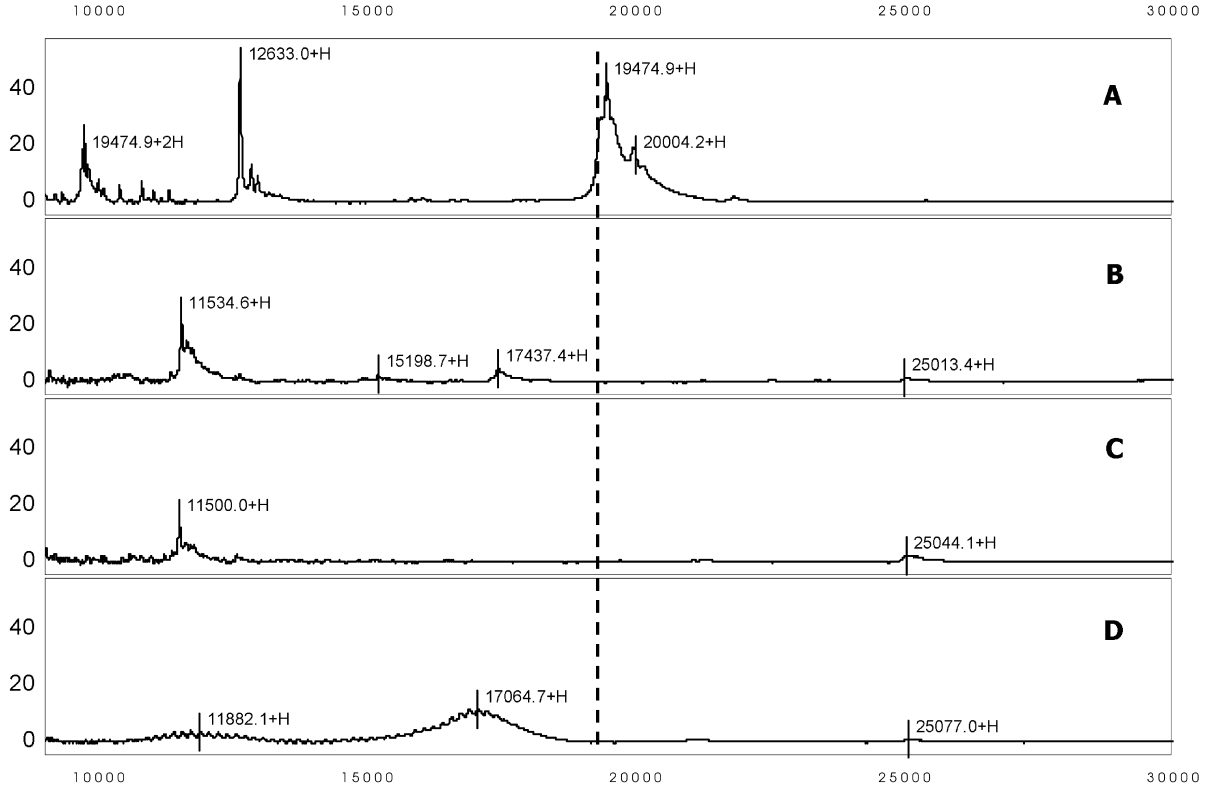

Fig. 1. Mass spectrometry results from different array surfaces loaded with a crude *Pichia pastoris* cell culture supernatant containing endostatin. (A) WCX2: weak cation-exchange surface in 50 m*M* acetate buffer, pH 5.0; (B) SAX2: strong anion-exchange surface in 50 m*M* Tris–HCl buffer, pH 9; (C) H4: hydrophobic interaction surface in 50 m*M* Tris–HCl buffer, 1000 m*M* sodium chloride, pH 7.5; (D) IMAC3: chelated surface with Cu^{+2} in 20 m*M* phosphate buffer, 500 m*M* sodium chloride, pH 7.0. Endostatin positioning is indicated by the dotted line.

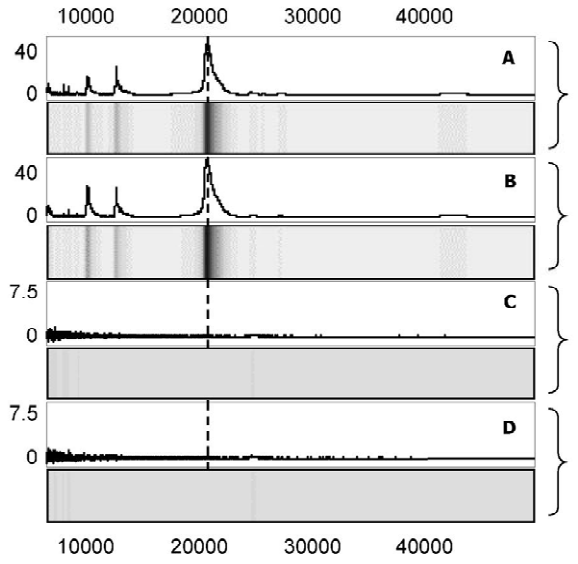

Fig. 2. Mass spectrometry results of endostatin elution from WCX 2 array loaded with a *Pichia pastoris* cell culture supernatant. Determinations were performed at constant pH of 5.0 and at four different ionic strengths using sodium chloride concentrations of 50 m*M* (A), 100 m*M* (B), 300 m*M* (C) and 700 m*M* (D). Endostatin positioning is indicated by the dotted line.

The evaluation of protein separation processes, using chip surfaces technology as an indication to separation on packed and fluidized bed columns, requires a continuity of the solid-phase chemistry. The same chemical group should be linked on the same synthetic polymer solidly anchored on the mineral surface. Since ion-exchange adsorption–desorption mechanisms of proteins to a porous planar surface or to a porous bead are strictly the same, any minor changes in the chemistry affect the separation optimization process. In the chip surface technology, the sample is loaded on the surface of the selected array in appropriate conditions of pH and ionic strength. A wash step follows to remove proteins that do not interact on the surface, in the same way as is done in column chromatography. Modifying the pH of the buffers and increasing the ionic strength typically result in different adsorption patterns. Unlike chromatographic separations, a laser beam accomplishes the elution of proteins on the surface in presence of energy adsorption molecules. The eluted proteins are analyzed in the time-of-flight mass spectrometer (TOF-MS).

3.2. Evaluating endostatin binding capacity to packed and fluidized cation-exchange columns

The data collected from chip surface interaction with endostatin, were used for developing an in-column process for endostatin capture. A sorbent with similar functionality must to be used, since it is assumed that adsorption–desorption mechanisms are the same on flat surface and on porous beads. CM HyperZ, a zirconium oxide porous beaded sorbent impregnated with a synthetic hydrogel, has identical chemical functionality to the chip planar surface. Therefore, it is expected that the material will adsorb and desorb endostatin in the same way as in a packed column. This material is of high density and applies particularly well to fluidized bed separations [16].

Once the resin was packed in the column (see Experimental section), a large volume of crude clarified cell culture supernatant was loaded into the column under an upward constant linear flow-rate of 535 cm/h. At the column outlet samples were periodically taken for endostatin analysis by mass spectrometry and for the determination of the specific breakthrough curve. This is an efficient approach to dynamic binding capacity measurement since it enables the specific determination of endostatin, and eliminates the cumbersome and time-consuming SDS–PAGE analysis.

Fig. 3 illustrates endostatin breakthrough curves determined with the effluents of both packed and fluidized bed column configurations. These breakthrough curves were built from peak intensity values of endostatin in the assayed fractions all along the chromatography, and expressed as a percentage of the intensity of endostatin peak in the crude supernatant. Binding capacity of endostatin at 10% break through was then calculated by the frontal analysis method previously described [17]. Found values were 14 and 13.7 mg endostatin per ml of resin, respectively in packed bed and in fluidized bed.

Table 2 shows clearly that the binding capacity for endostatin diminished when the flow-rate increased; this phenomenon is enhanced when short columns are being used. Combination of short columns and high flow-rates induced a significant decrease of the residence time and therefore reduced binding capacity. But, due to the relatively low molecular mass of the investigated protein, the impact of residence time

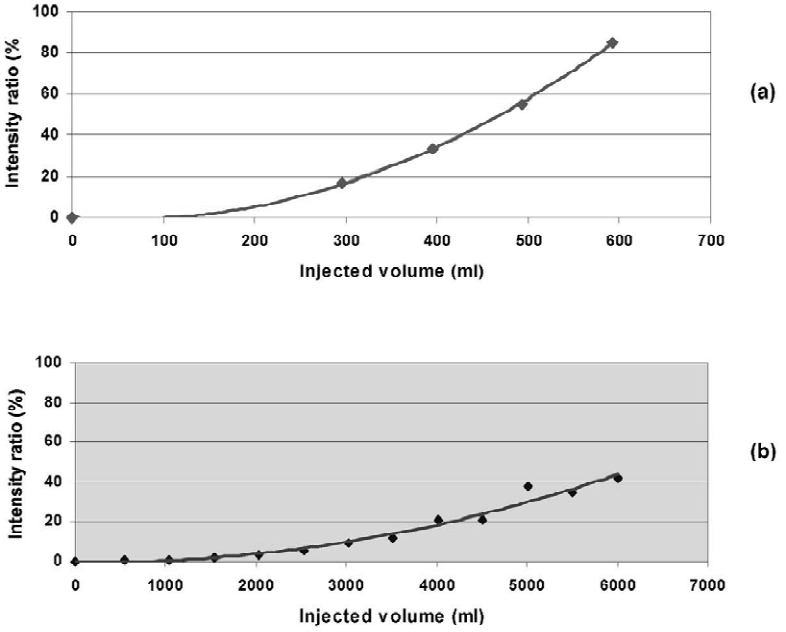

Fig. 3. Dynamic binding capacity of endostatin on packed and fluidised beds. Breakthrough curves were obtained by endostatin determination in the column effluents using mass spectrometry: *y* axis is intensity ratio in percentage between endostatin peak intensity in the collected fraction and endostatin peak intensity in the crude supernatant. (a) Curve from a packed bed column; (b) Curve from a fluidized bed column. Linear flow-rates were 300 and 350 cm/h, respectively. Buffer used was: 50 m*M* acetate, pH 5.0.

on binding capacity was limited to few mg/ml. It is important to indicate that binding capacity in fluidized bed mode was similar to that of the packed bed mode. As a result of column dimension and expansion factor, the residence time in the fluidized bed column was longer than the one in the packed bed column. With a fixed amount of solid-phase sorbent, a column diameter of 1 cm and an expansion factor of 2 corresponding to a flow-rate of 350 cm/h, the residence time was 3.4 min compared to 0.5 min for the packed bed described above.

The overall chromatography processes of the packed and fluidized bed columns are shown in Fig. 4. The chromatographic profiles were very similar and final purity of endostatin measured by SDS–PAGE was similar, in the range of 80-85% (data not shown) with recovery of 75–80%.

3.3. Pilot recovery of endostatin from complete Pichia pastoris culture

Once the characteristics of endostatin binding on fluidized bed were determined,capturing the target protein from complete fermentation broth was per-

Table 2
Dynamic binding capacity (DBC) of CM HyperZ for endostatin in packed bed mode starting from crude clarified *Pichia pastoris* supernatant

Column I.D. (mm)	Bed height (cm)	Flow rate (cm/h)	Residence time (min)	Volume injected (ml)	DBC at 10% breakthrough (mg/ml)
3.0	10	350	1.7	200	>8.6[a]
3.0	10	1028	0.58	741	13.7
6.6	2.1	300	0.42	800	12.9
6.6	2.1	1028	0.12	621	7.5

[a] 10% breakthrough was not reached.

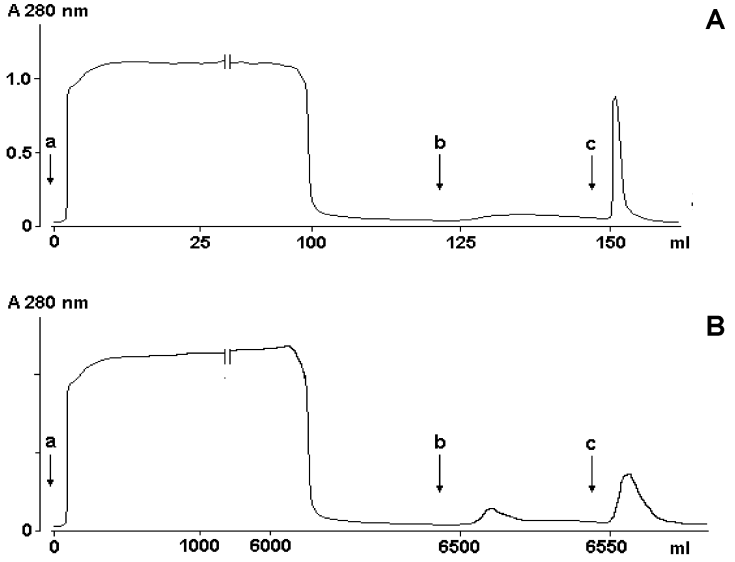

Fig. 4. Chromatographic profiles of endostatin capture from clarified *Pichia pastoris* cell culture supernatant using packed bed (A) and fluidized bed (B). a=sample injection; b=200 m*M* sodium chloride step; c=800 m*M* sodium chloride elution step. For operational details see Experimental section. The arrow indicates the elution of endostatin by a step of 800 m*M* sodium chloride in acetate buffer.

formed. Fig. 5 [5] schematically represents a solid-phase fluidized bed for protein capture as it was used in the present work. A 43 l volume of *Pichia pastoris* culture containing 14.3 kg of wet biomass and 3.7 g of endostatin was diluted 2.5 times to a final volume of 110 l; the pH was adjusted to 5.0 and the conductivity to 7.1 mS/cm. The complete suspension was pumped upward on 1500 ml CM HyperZ at a linear flow-rate of 535 cm/h. At this flow-rate the expansion factor was 3 and the residence time 6.4 min. Due to the large amount of sorbent used, the residence time was higher than what was obtained using the clarified feedstock in the initial studies with a small fluidized bed column.

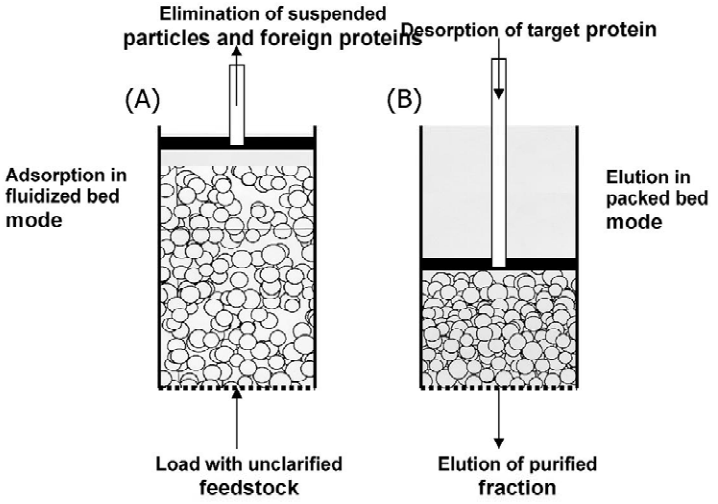

Fig. 5. Schematic representation of protein capture using expanded bed: equilibration, loading and washing steps are performed under expanded bed mode (A) by injecting the solutions upward; elution is then performed under packed bed mode (B) by reversing the flow.

Table 3
Endostatin capture using Zirconia based fluidized bed chromatography

Fraction	Volume (l)	Biomass concentration (g/l)	Protein (g/l)	Total endostatin (g)
Complete fermentation broth	43	332	5.7	3.7
Diluted fermentation broth	110	130	2.23	3.7
Column elution	1.0	0.0	5.54	3.17

Table 3 summarizes the main results of the operation where it appears that the amount of collected endostatin was 3.17 g corresponding to a recovery of about 85%. SDS–PAGE results in Fig. 6 demonstrate the efficiency of the separation. In the initial crude feedstock, the endostatin concentration was approximately 1.5% and the flowthrough fraction was depleted from endostatin while the elution fraction contained mainly endostatin. The endostatin concentration was approximately 60% achieving purification factor of approximately 40.

This work was limited to the early stage of the purification process, the capturing of endostatin from the crude culture broth. In this respect, the operation gave good results in terms of recovery and purification. Information collected from mass spectrometry indicated that it would be possible to eliminate some protein impurities on hydrophobic and on IMAC surfaces while leaving endostatin in the flowthrough. This information is of importance because these surfaces can be implemented for further purification steps to obtain a very pure preparation. Impurities detected by mass spectrometry, corresponding to molecular masses of 17 000 and 25 000 are still present in the partially purified endostatin.

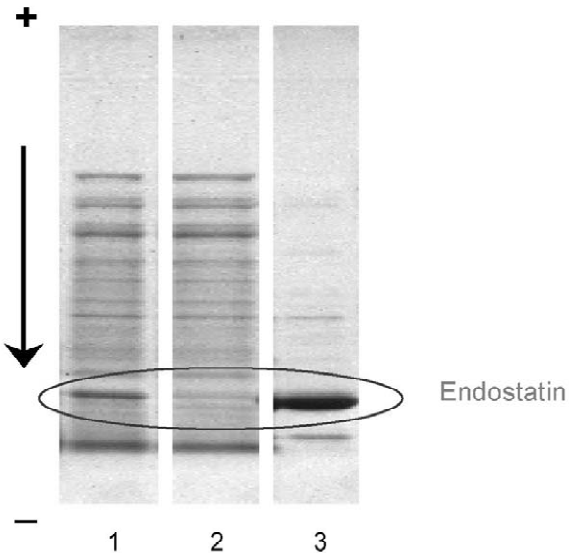

Fig. 6. SDS–PAGE of main endostatin capturing fractions from crude fermentation broth on fluidized bed. Lanes: 1, crude cell supernatant; 2, flow through from the fluidized bed column; and 3, eluted fraction from the column.

Investigations of the whole separation process are in progress.

4. Conclusion

Endostatin is a recombinant protein intended for therapeutic use and as such it must be purified to homogeneity taking costs into consideration. Currently, the protein is recovered not only from large amounts of contaminated proteins but also from large amount of biomass, which constitutes 30–50% of the total fermentation volume.

The process described here demonstrates that it is possible to optimize a process using non-conventional methods that guarantee the efficiency of the process and reduces significantly the process development time. It is also demonstrated that the separation conditions defined and optimized using selected arrays, can be directly applied to the fluidized bed separation process. Once the conditions of adsorption and desorption are optimized, the only missing information is the binding capacity of the target protein from the crude feedstock. Successful operation requires that the resin volume and the flow-rate will provide an expansion factor between 2 and 3. To minimize overloading, with consequent losses of desired product, the volume of the resin must exceed the theoretical volume.

Following the capture steps, the polishing stages for the final purification of the target protein remain

to be defined, and the mass spectrometry analysis gives indications of possible direction.

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