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Continuous-bed chromatography for the analysis and purification of recombinant human basic fibroblast growth factor

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Abstract

The chromatographic properties of the commercial cation exchanger UNO-S1 (35×7 mm) was investigated using lysozyme from hen egg white as model protein and recombinant human basic fibroblast growth factor (rh-bFGF) from a high cell density cultivation of *E. coli*. The dynamic capacity for lysozyme ($c°$=1 mg/ml) in 100 m*M* acetate buffer, pH 5 was 27 mg per ml sorbent. It was found independent of the flow-rate from 78 to 935 cm/h owing to the absence of mass transfer restrictions with this column concept. Regarding the selectivity for rh-bFGF and the capacity for lysozyme, no changes were apparent after cleaning-in-place (CIP) procedures with 0.5 *M* NaOH. Clogging of the column by a clarified crude cell homogenate of *E. coli* was not critical as precipitates were removed by reversal of the flow during CIP. Rh-bFGF elutes in three consequent peaks from the UNO-S1 column, which could be attributed to soluble rh-bFGF aggregates of different size. The dynamics of rh-bFGF aggregation and reaggregation in the crude feedstock was monitored by fast gradient elution chromatography. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Growth factors; Continuous beds; Proteins

1. Introduction

Downstream processing of recombinant proteins holds a key position in the production and purification of biopharmaceuticals. Regarding efficiency and productivity of single purification steps it is a major requirement to monitor the target protein in its biologically active form [1].

Apart from selectivity, robustness and speed are decisive criteria for the selection of analytical methods, which are to be employed for this purpose.

In the past years, new media concepts were developed to increase speed at analytical and preparative scale. Consequently, gel in a shell [2], superporous [3], perfusive [4] and membrane-based [5] materials were introduced to the market [6–8]. Among these, a novel column concept is based on a continuous column bed, which is made by in-column polymerization [9]. The structure of the column bed is best circumscribed by a thick microfiltration membrane. The term continuous-bed chromatography originates from the production of this unique stationary phase by polymerization in the column, thereby forming one single bead. With such an one-

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block-polymer mass transport resistances ought to be absent, so that the resolution becomes independent of the flow-rate, similar to membrane-based chromatographic supports [8].

As part of a process for the purification of recombinant human basic fibroblast growth factor (rh-bFGF) involving expanded bed adsorption (EBA), fast analysis of the feedstock and eluates from EBA was necessary to monitor the concentration of rh-bFGF and the dynamics of aggregation and deaggregation during purification. Starting point was a homogenized feedstock from a high-cell-density cultivation of *E. coli* containing a concentrated solution of hundreds of proteins with high viscosity owing to the released DNA fragments. Clogging of chromatographic columns with time can be expected after application of the crude solution even after removal of cells and cell debris by centrifugation or filtration, as always some smaller cell debris will remain. This is especially crucial for chromatographic materials with narrow particle size or membranes.

It was investigated in this study whether the concept of continuous-bed chromatography can be applied for the analysis and purification of rh-bFGF originating from the supernatant of a crude microbial broth. No particular attention was paid to the membrane-type characteristic of the column, which should be prone to clogging and fouling when applying cloudy fluids. Routine cleaning-in-place (CIP) procedures were applied, as is required for a validated process, and the column was treated to the limits regarding flow-rate and column pressure. The stability of the column was examined in view of the reproducibility of total lysozyme binding capacity and rh-bFGF retention times.

2. Experimental

2.1. Materials

Chicken egg white lysozyme (EC 3.2.1.17; M_r 14 100; isoelectric point, pI=11) and iodoacetamid were obtained from Sigma (Steinheim, Germany), DNase was from Boehringer Mannheim (Mannheim, Germany). Analytical-grade sodium hydroxide, sodium hydrogenphosphate, sodium acetate and 1,4-dithiotreitol were purchased from E. Merck (Darmstadt, Germany), Coomassie blue G 250 and trichloroacetic acid from Fluka (Buchs, Switzerland). Streamline SP was purchased from Pharmacia Biotech (Uppsala, Sweden).

2.2. Instrumentation and column

Chromatography was carried out on a fast protein liquid chromatography (FPLC) system including two P-500 pumps, a controller LCC-500 Plus, a fraction collector Frac-100 (Pharmacia Biotech) and a UV detector (280 nm) UV 2510 Uvicord SD (LKB, Bromma, Sweden).

A UNO-S1 column (35×7 mm) with strong cation-exchange sulfopropyl (SP) functional groups was kindly donated by Bio-Rad (Munich, Germany). The chromatographic column contains an in-column polymerized bed of the copolymer of acrylic acid and *N*,*N*′-methylenebisacrylamide [9].

2.3. Methods

2.3.1. Production of rh-bFGF

rh-bFGF was produced in high cell density cultivation of *E. coli* TG1:pλFGFB [10]. After cell harvesting, soluble rh-bFGF was released by bead mill cell disruption. The resulting cell homogenate was stored at −20°C until used.

2.3.2. Chromatographic conditions

Frontal breakthrough curves were obtained with feeds consisting of 1.0 mg/ml lysozyme in 100 m*M* sodium acetate buffer (pH 5) at flow-rates of 0.5, 3 and 6 ml/min, corresponding to 78, 468 and 935 cm/h, at ambient temperature (20±2°C). Elution was done with a linear gradient of up to 1.5 *M* sodium chloride in an acetate buffer and operating at a gradient steepness corresponding to 10 column volumes.

The homogenized rh-bFGF solution was incubated for 30 min with 250 μg DNase per gram wet biomass to reduce the viscosity. For the analysis of rh-bFGF the suspension was centrifuged for 30 min at 40 000 *g* and the pH and conductivity were adjusted according to the column equilibration buffer (0.05 *M* sodium phosphate+0.15 *M* sodium chloride, pH 7, conductivity 18.6 mS/cm). After loading 3 ml of the clarified homogenate, the column was washed

with 5.5 column volumes equilibration buffer before starting the elution with a linear gradient of up to 1.5 *M* sodium chloride (pH 7) in a phosphate buffer and a gradient steepness corresponding to 15 column volumes. After elution the column was re-equilibrated with 14 column volumes. All buffers were filtered through 0.2-μm cellulose nitrate filters (Minisart NML, Sartorius, Germany) before usage.

2.3.3. Cleaning-in-place (CIP) procedure

After three injection/elution cycles of the clarified crude feedstock rh-bFGF solution the column was cleaned with 4.5 column volumes of 0.5 *M* sodium hydroxide at a flow-rate of 1 ml/min. Afterwards re-equilibration was done with 11 column volumes of equilibration buffer.

2.3.4. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a Multiphor II unit with a power supply EPS 3500 XL using precast ExcelGel SDS gradient 8–18% gels and buffer strips, all from Pharmacia Biotech. Sample treatment under reducing and non reducing conditions as well as staining with Coomassie blue G 250 and silver was carried out as recommended by the supplier, according to Refs. [11,12]. Alkylation of free –SH groups of proteins with iodoacetamid and protein precipitation with trichloroacetic acid was done as described [13].

2.3.5. Scanning electron microscopy (SEM)

A small amount of UNO-S1 material was removed from the column, air dried and then sputtered with gold in a sputter unit SCD 040 (Balzers Union, Liechtenstein). Scanning electron micrographs were obtained at 1.00 kV using a Zeiss DSM 982 Gemini (Oberkochen, Germany).

3. Results and discussion

3.1. Morphology of column bed

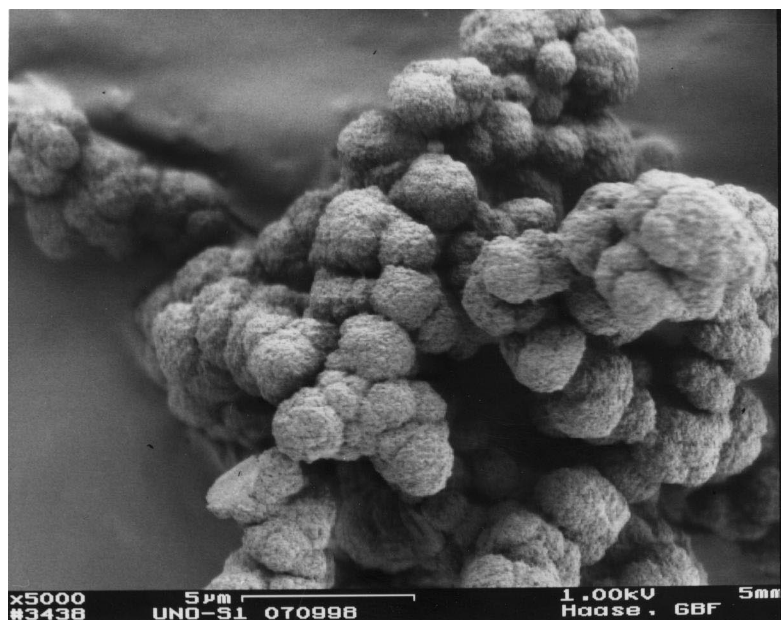
The process of in-column polymerization provides a polymer network in the column, as can be seen in the SEM photograph of a sample taken from the column top (Fig. 1a). The particle-like structure, showing diameters from 2 to 3 μm, is composed of small interconnected non-porous nodules. According to the manufacturer, the size of these nodules is about 0.2 μm. Also the larger particles are interconnected, thereby forming open channels. This is shown in the schematic representation in Fig. 1b, which is adapted from Liao et al. [14].

Although the sample from the column bed was prepared using the critical point method, it should be emphasized that the morphology of the bed in the column may differ from that in the sample. Accordingly, it is uncertain whether the channels observed in Fig. 1a have the same dimensions as in the column. However, the general structure explains many properties of the UNO column. These channels are large enough to allow high flow-rates of up to 935 cm/h without notable compression of the column bed.

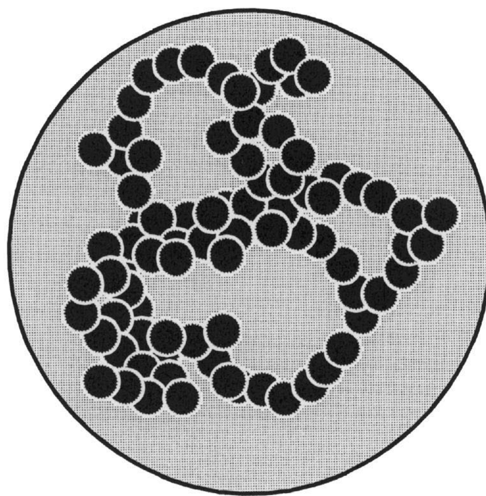
3.2. Frontal chromatography of lysozyme

According to Fig. 2, shape and position of lysozyme breakthrough curves are almost identical in the range of flow-rates chosen (78 cm/h, 0.5 ml/min; 468 cm/h, 3 ml/min; 935 cm/h, 6 ml/min). Thus, a change in the dynamic capacity of the UNO-S1 column is not significant over this range of flow-rates. The back pressure increased linearly with the flow-rate to 3.5 MPa at 935 cm/h, which is the highest possible flow-rate recommended by the manufacturer. The steep and uniform breakthrough curves at $c/c^{\circ}<0.8$ are indicative for a fast mass transport in the column. So it follows that mass transport limitations, e.g., pore and film diffusion, indeed are of minor relevance with this column concept. The moderate incline at $c/c^{\circ}>0.8$ is related to steric effects, caused by desorption and rearrangement of adsorbed proteins, which can be described with the random site surface (RSS) model [15].

Similar protein binding capacities than that of the single bead adsorber are found with membrane adsorbers based on microfiltration membranes, which range between 10 and 40 mg/ml, depending on the protein and the adsorption conditions [16,17]. In contrast, the equilibrium capacity per column volume of Streamline is higher, which is due to the contribution of the inner surface area of diffusion pores. Also the dynamic capacity at $c/c^{\circ}=0.2$ of the expanded-



(a)



(b)

Fig. 1. (a) Scanning electron micrograph of UNO-S1 material scraped of the column top. (b) Schematic representation of the UNO-S1 support.

bed adsorber is higher; however, it decreases from 100 to 60 mg/ml (at $c°=4$ mg/ml) with increasing the flow-rate from 25 to 250 cm/h (300 cm/h is the recommended flow rate according to the manufacturer) in a packed-bed mode (data not shown). The dynamic capacity at $c/c°=0.2$ (at $c°=1$ mg/ml) of

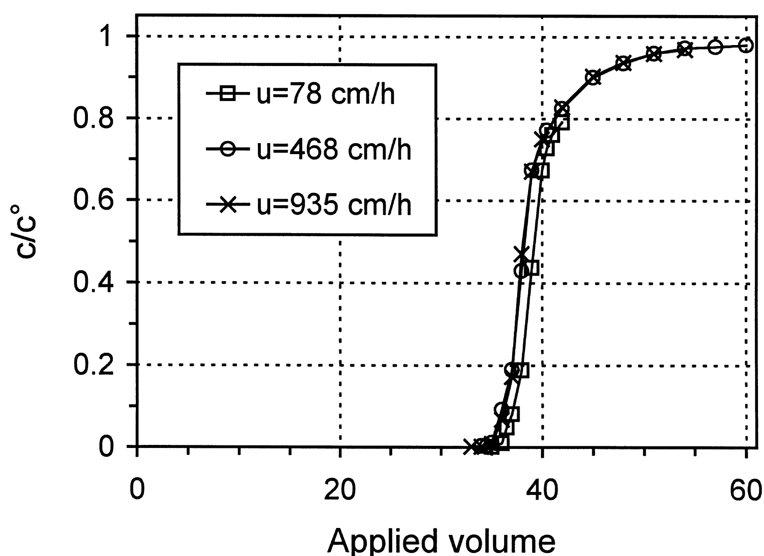


Fig. 2. Breakthrough curves of lysozyme on a UNO-S1 column (35×7 mm) at different flow-rates; 1 mg/ml lysozyme was dissolved in 100 m*M* sodium acetate buffer, pH 5.

the UNO-S1 column is constant up to the highest possible flow-rate, corresponding to 27 mg lysozyme per ml column. However, one should keep in mind that the lower equilibrium capacity of the UNO-S1 column is compensated by the higher possible flow-rate without loss in dynamic capacity. Although practically of minor relevance, the protein throughput would be even higher compared with the expanded-bed adsorber if elution cycles are ignored. However, this is an oversimplification; taking into account elution and regeneration cycles, comparable protein throughputs are reached.

3.3. Process analysis with the UNO-S1 column

To evaluate purification efficiency and protein recovery during processing the feedstock from *E. coli* cultivation, the breakthrough of rh-bFGF on the column used for product analysis must be minimized. Therefore, different volumes of the clarified crude homogenate were loaded on the UNO-S1 column. Owing to the strong interactions with the cation exchanger, up to 20 ml could be loaded before a measurable amount of rh-bFGF was detected by SDS–PAGE (data not shown). Consequently, for further chromatographic analysis the injection volume was reduced to 3 ml. In comparison to the elution profile from the expanded-bed adsorber in a packed-bed mode (56×5 mm), rh-bFGF elutes in three consequent peaks from the UNO-S1 column with increasing salt concentration (Fig. 3). This can be attributed to a higher number of theoretical plates of the UNO-S1 column in comparison to the packed-bed. Both under reducing (inset of Fig. 3) and non reducing (data not shown) conditions these chromatographic peaks show only one band for rh-bFGF in SDS–PAGE, while the band of a contaminating protein with smaller molecular mass is only found at peak positions 1 and 2. The latter corresponds to histone-like (HU) protein [18,19], as identified by N-terminal sequence analysis. It is a DNA-binding protein with a p*I* between 9.6 and 9.7 (SWISS-PROT data bank, P02341/42). This is very close to the p*I* of rh-bFGF (p*I* 9.6, [20]), which is the reason that the two proteins could not be separated by cation-exchange chromatography.

Re-chromatography of each peak yielded the same elution pattern with varying peak intensities, with the peaks attributed to the monomer and different aggregation states of rh-bFGF. The process of aggregation and dissociation is probably to fast to allow separation of aggregates by SDS–PAGE but not fast enough to prevent their separation on the time scale of the chromatographic analysis. To prove this

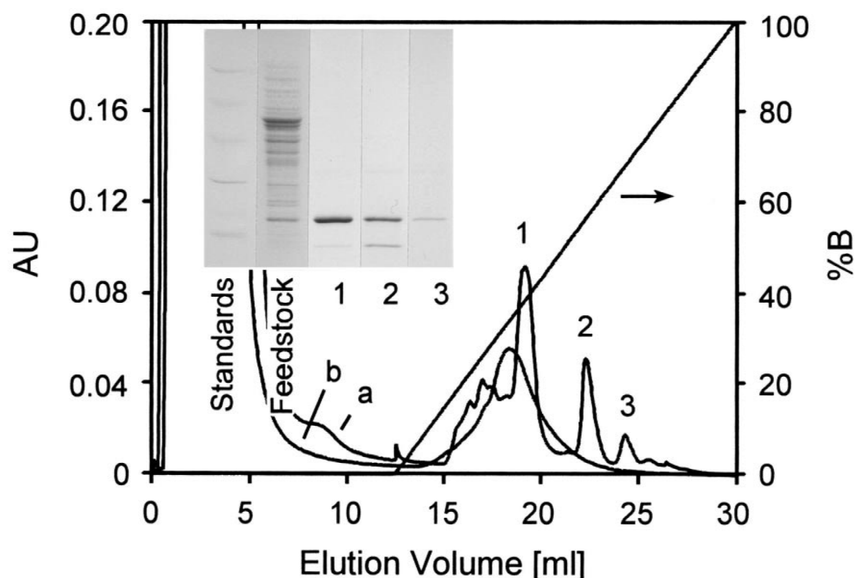


Fig. 3. Separation of 3 ml clarified crude feedstock on UNO-S1 (line a, 624 cm/h) and Streamline SP (line b, 150 cm/h, packed-bed mode) in 50 m*M* sodium phosphate+0.15 *M* sodium chloride, pH 7. Linear elution was performed with 50 m*M* sodium phosphate+1.5 *M* sodium chloride, pH 7 (buffer B). Inset: SDS–PAGE gels with molecular mass standards (14 000, 20 000, 30 000, 43 000, 67 000 and 94 000), clarified rh-bFGF feedstock and peaks 1 to 3 under reducing conditions.

hypothesis, highly purified rh-bFGF was injected and the eluted peak fractions were alkylated before SDS–PAGE. This chemical modification assures that free S–H groups of rh-bFGF can no longer take part on a quick S–S bond exchange, a process which is known to take place with this protein [13] and which is characteristic for this type of proteins [21]. After this modification the different aggregates of rh-bFGF were also separated by SDS–PAGE and up to tetramers were established (Fig. 4).

To follow aggregation and dissociation of rh-bFGF in the crude feedstock, repeated injections were performed on the UNO-S1 column over several hours. Peak areas of the different forms of rh-bFGF changed over time with the monomer increasing at the expenses of higher aggregation states (Fig. 5). However, the total rh-bFGF concentration remains constant, indicating that protein precipitation does not take place. This result demonstrates that process analysis with the UNO-S1 column provides a convenient method to look into the time-dependent aggregation and re-aggregation of rh-bFGF during purification and to optimize processing of the crude feedstock regarding the monomer separation.

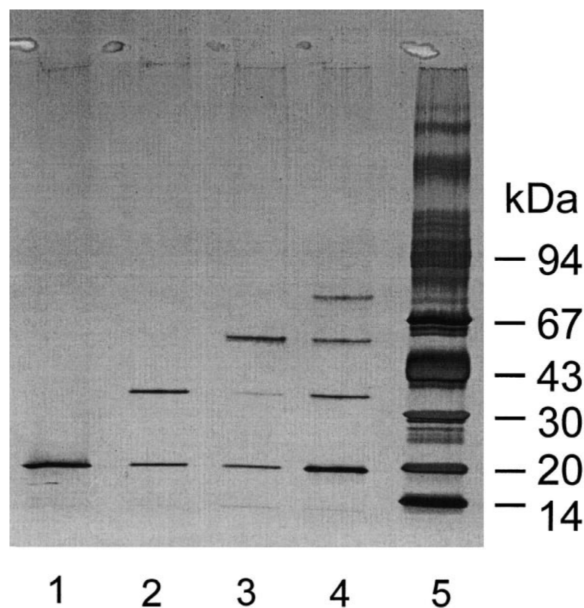


Fig. 4. SDS–PAGE of rh-bFGF fractions eluted in four consequent peaks from UNO-S1 column under non reducing conditions (lanes 1–4) and molecular mass standard (lane 5; kDa= kilodalton). Samples were treated with iodoacetamid for alkylation of free –SH groups as described [13].

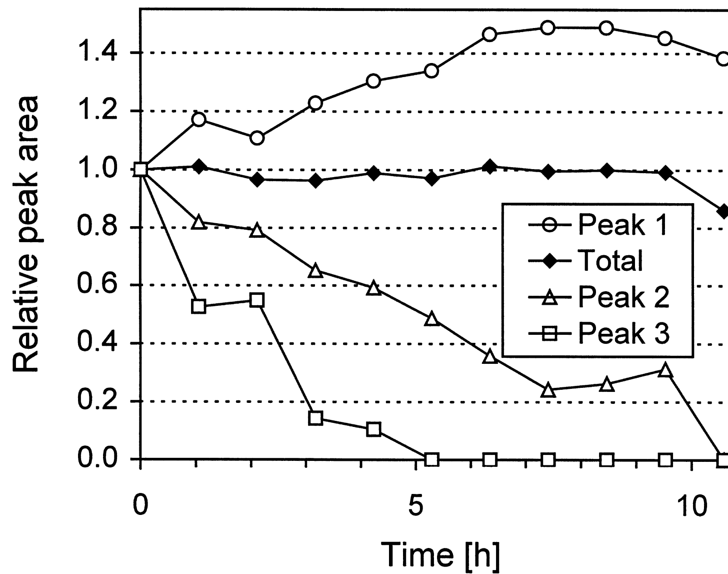


Fig. 5. Time course of relative peak areas of peaks 1, 2 and 3 (Fig. 3) from clarified rh-bFGF cell homogenate at room temperature.

3.4. CIP stability and fouling

Under alkaline CIP conditions using 0.5 *M* NaOH, scattering of retention times of the three rh-bFGF peaks were insignificant in view of measurement errors (Fig. 6). Furthermore, the shape and position of lysozyme breakthrough curves after CIP procedures did also not change compared to Fig. 2. Therefore, neither a loss in selectivity nor in capacity was observed. During the time course of process analyses (33 injections, each 2.3-times the column volume of a clarified rh-bFGF feedstock), the column

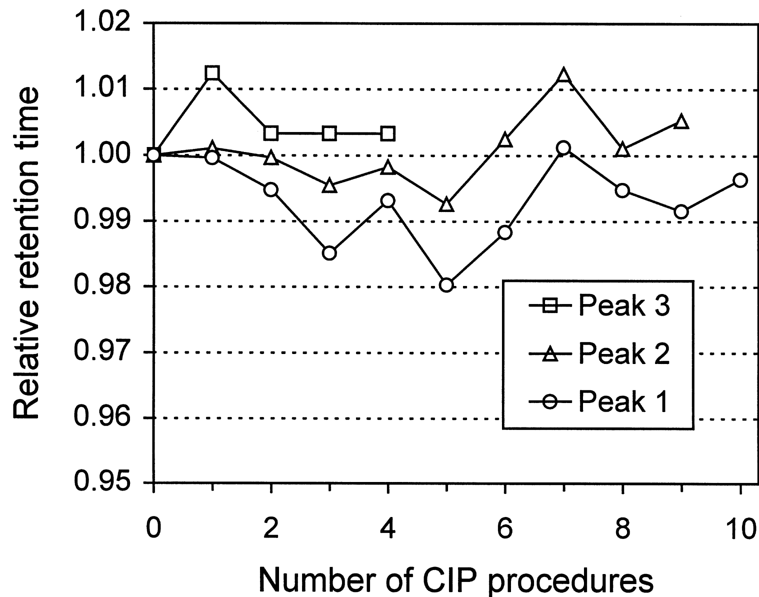


Fig. 6. Relative retention times of peaks 1, 2 and 3 as eluted from the UNO-S1 column (Fig. 3) after CIP procedures with 0.5 *M* NaOH.

back pressure raised up by 0.5 MPa. This was attributed to fouling at the column inlet. By inversion of the flow and employing 0.5 *M* NaOH, the precipitate was removed and the column back pressure recovered.

4. Conclusions

Continuous-bed chromatography using a UNO-S1 column allows fast gradient elution of proteins from a crude feedstock of a high density *E. coli* cultivation. Although the supernatant of the homogenate was still cloudy from small cell debris and insoluble protein aggregates, a complete blockage of the column did not take place. Fouling could easily be reversed by the application of 0.5 *M* NaOH. The routine application of this CIP procedure did affect neither the selectivity for rh-bFGF nor the binding capacity with using lysozyme as a model protein.

Owing to the unique structure of the column bed, mass transport takes place mainly by convection. Therefore, breakthrough curves and chromatographic peaks were almost independent of flow-rates up to 935 cm/h, which is the upper limit recommended by the manufacturer. The protein binding capacity is lower compared to conventional porous chromatographic media. However, the lower binding capacity can be compensated by fast flow-rates that can be combined with a fast cycle technique.

The fast gradient elution chromatography allows to follow the fast dynamics of protein aggregation and dissociation, as observed during the purification of rh-bFGF. Though not tested, of course this might also be realized by other high-performance supports, such as those employed in high-performance liquid chromatography (HPLC). However, this does not impair the generally good performance of this column concept introduced by Hjertén et al. [9].

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