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Journal of Chromatography A, 711 (1995) 129–139

JOURNAL OF CHROMATOGRAPHY A

Purification of recombinant human basic fibroblast growth factor: stability of selective sorbents under cleaning in place conditions

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Abstract

Human basic fibroblast growth factor (bFGF) was produced from recombinant *Escherichia coli* by high-cell-density cultivation. In order to develop a purification strategy for large-scale purification, chromatographic sorbents with different anionic functional groups were compared in terms of selectivity for bFGF and stability under cleaning in place (CIP) conditions. Heparin-Sepharose CL-6B, Fractogel EMD-SO_3^- 650 (S) and SP-Sepharose (high performance) were found suitable for this purpose with decreasing selectivity in that order. Each sorbent was treated eight times under CIP conditions employing both 0.2 and 1.0 *M* NaOH, in order to study modifications of these sorbents. Heparin-Sepharose displayed more than 50% loss of capacity after the first CIP treatment and decreasing selectivity with each cycle. Both cation exchangers displayed almost constant results regarding selectivity and capacity. The Fractogel EMD-SO_3^- exhibited only slightly lower selectivity for bFGF than Heparin-Sepharose and the highest capacity of all sorbents tested. Agglomeration of bFGF at low salt concentrations was a serious problem. By direct application of pooled fractions from Fractogel EMD-SO_3^- onto Heparin-Sepharose a highly pure product was obtained; however, the recovery after Heparin-Sepharose was only 30%.

1. Introduction

Basic fibroblast growth factor (bFGF) controls different functions in eucaryonts. It is an important factor during embryonal development, it stimulates mitosis of different types of cells, such as fibroblasts, and it enhances angiogenesis. The protein has profound interests in the pharmaceutical industry due to several potential applications, such as wound healing and redirection of blood vessels.

The purification of bFGF from natural biological sources, such as blood and human vascular smooth muscle cells, is described in numerous publications [1–3]. Since the concentration of bFGF in these sources is generally low (between 10^{-9} and 10^{-11} *M*), mostly small amounts of this protein were isolated from large volumes containing other proteins in much higher concentrations. Thus, highly specific affinity chromatographic sorbents, such as Heparin-Sepharose, are commonly employed for this purpose [4,5].

Major advances in gene technology in the past years made it possible that an increasing amount of pharmaceutically important proteins are produced by recombinant microorganisms [6,7]. The concentration of the recombinant protein in the

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SSDI 0021-9673(95)00102-6

culture broth is often higher than that of the genuine protein, even by circumventing the development of inclusion bodies. Utilising special techniques, such as high-cell-density cultivation, very high concentrations of recombinant protein can be achieved in the soluble form [8].

Human bFGF can be expressed in recombinant *Escherichia coli* [9,10]. In contrast to the low concentration of bFGF in natural sources, the concentration of soluble recombinant protein was found between 2.3 and 3.4 g/l after high-cell-density cultivation [11]. Nevertheless, the clearance of host-specific proteins demands for selective sorbents, since high purity of the product is a major constraint for in vivo applications of recombinant proteins [12].

The applicability of bFGF-selective sorbents for the large-scale purification was the object of this investigation. Focal points were the selectivity for bFGF, in order to keep down the number of different chromatographic steps during downstream processing, and the stability of suitable sorbents under cleaning in place (CIP) conditions [13]. CIP is routinely employed in the large- and process-scale purification of proteins in order to avoid contamination of the chromatographic equipment with microorganisms. Even small numbers of microorganisms are crucial if proteins for pharmaceutical applications are purified due to contamination of the product with pyrogens [14,15].

Affinity sorbents, as obtained through immobilization of proteins such as immunoglobulins, lectins or protein A, denature under these harsh chemical conditions; at 1 *M* NaOH hydrolysis of the peptide chain is expected to take place. Although heparin sorbents are effective for the isolation of bFGF from different tissues they are not stable under these CIP conditions; thus, this is not recommended by the manufacturer [16]. Heparin is a group-specific ligand which displays interactions with many proteins [17,18]. Negatively charged functional groups of different chemical structure along the carbohydrate backbone of heparin are mainly responsible for these ionic interactions (Table 1). Sorbents consisting of anionic polymeric ligands or cation exchangers consisting of flexible polymeric chains were likely to interact in a mode similar to heparin. Such sorbents were studied for their selectivity against bFGF. The most suitable sorbents were then investigated more closely for their stability against CIP conditions in terms of changes in capacity and selectivity.

Table 1
Functional groups of sorbents employed for the purification of bFGF

Sorbent	Ionic group	Gradient
Heparin-Sepharose CL-6B	$OSO_3^- \cdot NHSO_3^- \cdot COO^-$	0.6–2.0 *M* NaCl in 30 min
Fractogel EMD-SO_3^- 650 (S)	SO_3^-	0.6–1.6 *M* NaCl in 60 min
SP-Sepharose (HP)	SO_3^-	0.2–1.0 *M* NaCl in 60 min
Single-stranded DNA–Agarose	PO_4^-	0.2–1.0 *M* NaCl in 30 min
Polyadenylic acid–Agarose	PO_4	0.2–1.0 *M* NaCl in 30 min
Poros 20 HS	SO_3^-	0.2–2.0 *M* NaCl in 5 min

20 m*M* Tris–HCl, pH 7.4 was used in all experiments. Salt concentration in the equilibration buffer and linear gradients as described.

2. Experimental

2.1. Chemicals and chromatographic sorbents

Heparin-Sepharose CL-6B and SP-Sepharose (high performance) were purchased from Pharmacia (Freiburg, Germany). Fractogel EMD-SO_3^- and dithiothreitol (DTT) were obtained from E. Merck (Darmstadt, Germany). Polyadenylic acid–agarose and mercaptoethanol were obtained from Sigma (Munich, Germany). DNA–agarose was purchased from Life Technologies (Eggenstein, Germany). Poros 20 HS was obtained from PerSeptive Biosystems (Freiburg, Germany). Lysozyme from hen egg white was obtained from Fluka (Neu-Ulm, Germany). Buffer salts and other chemicals were from Riedel-de Haen (Seelze, Germany). HPLC-grade acetonitrile was obtained from J.T. Baker (Gross-Gerau, Germany).

bFGF was produced in high-cell-density cultures of recombinant *E. coli* TG1:pλFGFB [11]. Soluble bFGF was found in the supernatant remaining after cell homogenization and centrifugation in 0.1 *M* phosphate buffer, pH 7.

2.2. Instruments

The liquid chromatographic system used for all experiments was the Pharmacia 500 system assembled for zonal and frontal chromatography, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the 'Minigel' from Biometra (Göttingen, Germany). Protein concentrations were measured directly at 280 nm using the Perkin-Elmer 552 spectrophotometer and by quantifying stained protein bands from SDS-PAGE gels by densitometry (Elscript 400, Hirschmann, Germany). Measurements of circular dichroism (CD) were performed on the Jasco J-600 spectrophotometer (Biotronik, Frankfurt, Germany).

Thermodynamic investigations were performed using a LKB microperpex peristaltic pump, a variable-wavelength monitor from Knaur (280 nm, 10 μl cell volume) and a chart recorder (BBC Metrawatt, SE 120).

2.3. Chromatographic conditions

All sorbents were packed in water. Chromatographic experiments, including CIP procedures, were carried out using Pharmacia HR 5/5 columns of 5 mm I.D. and bed heights of approximately 50 mm. All chromatographic experiments were performed in 20 m*M* Tris–HCl, pH 7.4. Experiments regarding selectivity differences were carried out at 298 K at a flow-rate of 0.4 ml/min, except the Poros 20 HS support which was employed at a flow-rate of 5 ml/min.

The large-scale experiment was carried out using Pharmacia XK 16 (16 mm I.D.) with 50 mm and 83 mm column bed heights for Fractogel EMD-SO_3^- 650 and Heparin-Sepharose CL-6B, respectively. These columns were run at 4 ml/min.

2.4. Gradient elution of bFGF

All chromatographic experiments were performed using two pumps and a high-pressure mixing valve, as commonly assembled for zonal chromatography. The experiments were operated with automatic injection and elution. Columns were equilibrated with eight column volumes, and a crude sample containing approximately 1.7 mg/ml bFGF was injected using a 500-μl sample loop. Then the columns were washed with eight column volumes of equilibration buffer. Elution of adsorbed proteins was forced by a NaCl gradient. The different functional groups possessed by each sorbent and the relative chromatographic conditions employed for the purification of bFGF are reported in Table 1. Column effluents were monitored at 280 nm. Collected fractions were analyzed electrophoretically.

2.5. Frontal chromatography of lysozyme

Frontal chromatography was performed with automated adsorption, elution and equilibration.

Each run was terminated by the integrator after reaching the plateau region of the frontal breakthrough. To allow adsorption at defined conditions, lysozyme was dissolved in 20 m*M* Tris–HCl + 0.2 NaCl, pH 7.4. Elution of the protein was achieved by addition of 1 *M* NaCl. During stability tests, an additional CIP was placed between each elution and adsorption step.

2.6. Purification of bFGF

In order to get bFGF of the highest possible purity, two chromatographic steps were coupled in series. The sample volume was 45 ml with a bFGF concentration of 1.7 mg/ml. Fractogel EMD-SO_3^- was employed first to separate the major contaminants. Fractions containing bFGF were than directly adsorbed onto Heparin-Sepharose. Chromatographic conditions were identical to those described above. Fractions containing bFGF were dialysed against 20 m*M* phosphate buffer, pH 7.4 for 24 h at 4°C, unless stated otherwise. At last the bFGF was lyophilized for 36 h.

2.7. Cleaning in place procedures

Two CIP procedures with different concentration of NaOH were employed to account for different requirements reported in the literature (Fig. 1). 0.2 *M* NaOH was applied, as this treatment is recommended for the clean-up of *E. coli* [19]. 1 *M* NaOH is recommended for deactivating more resistant microorganisms, such as *Pseudomonas aeruginosa*. Under such strong alkaline conditions hydrolysis of proteins takes also place. Thus, the last procedure facilitates the removal of irreversibly adsorbed or precipitated proteins.

2.8. SDS-PAGE

Fractions were lyophilized and analyzed in SDS on 15% cross-linked polyacrylamide gels employing standard operating conditions [20]. Protein bands were visualized by silver staining [21]. Staining with Coomassie Blue was employed for quantitative analysis of protein concentrations [22] and compared to the method of Lowry et al. [23].

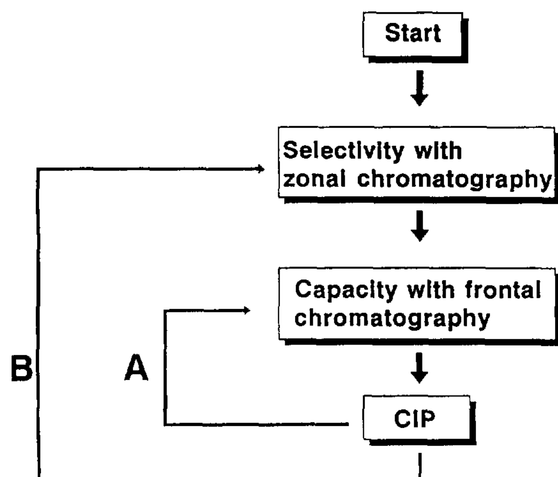

Fig. 1. Sequence of cleaning in place procedures and chromatographic tests as applied during stability experiments of affinity sorbents. Capacity was checked after each CIP, selectivity after the first, second, fourth and eighth CIP.

2.9. Determination of thermodynamic data

The experimental set-up illustrated in Fig. 2 and lysozyme as model protein was employed in

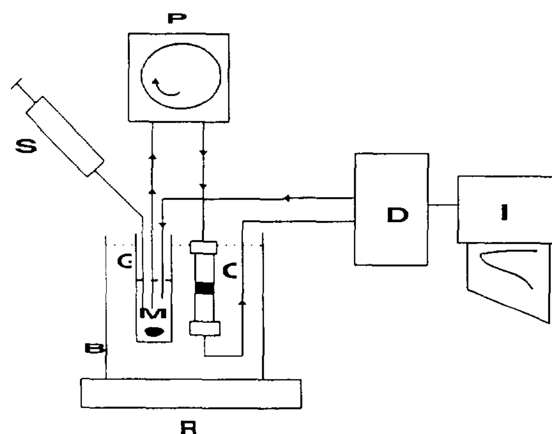

Fig. 2. Schematic diagram of experimental set-up for the determination of thermodynamic data (total hold-up volume at start was 3 ml). B = thermostatic bath, S = HPLC syringe, R = magnetic stirrer, M = magnet, G = continuously stirred tank, C = column (bed volume ≈ 40 μl), P = peristaltic pump (flow-rate 0.39 ml/min), D = detector, I = chart recorder.

all experiments. Assuming single-site interaction of both ligand and solute the accessible ligand concentration, q_m, and the apparent dissociation constant, K_D, were determined for Heparin-Sepharose, Fractogel EMD-SO_3^-, and SP-Sepharose. Using two adjustable column adapters the bed volume was set to 40 μl. The stirred tank and the column were placed into a thermostated bath at 298 K. A peristaltic pump continuously withdrew samples which were pumped through column and detector back into the tank at a flow-rate of 0.39 ml/min, in order to keep the volume constant. The detector was calibrated for lysozyme at different concentration to allow accurate measurement of absolute protein concentrations. A 3-ml volume of buffer was put in the stirred tank at the beginning and circulated through the system. After steady state was reached, a protein sample of known concentration and volume was injected by a HPLC syringe. The equilibrium concentration was recorded and subtracted from the injected protein mass to yield the adsorbed protein mass. In this manner the equilibrium isotherm was attained. In case of a Langmuir-type isotherm, the double-reciprocal plot of $1/q^*$ against $1/c^*$ yields a linear plot. This numerical approach permits q_m to be evaluated from the y-intercept and K_D from the slope of the linear plot [24,25].

3. Results and discussions

Polyadenylic acid–agarose and single-stranded DNA–agarose were not effective in the purification of bFGF. Both sorbents adsorbed proteins at NaCl concentrations of 0.2 M at maximum and displayed only one major peak at slightly higher salt concentrations containing bFGF and most of the host-specific proteins. This result differs from the isolation of some other proteins that were purified both with Heparin-Sepharose and single-stranded DNA [26,27]. In the case of bFGF a distinct adsorption seems to take place. Since no further optimization was achieved, these sorbents were disregarded in the following study.

Good purification levels were achieved using Heparin-Sepharose, Fractogel EMD-SO_3^-, and SP-Sepharose (Fig. 3). On Heparin-Sepharose bFGF eluted approximately at 1.8 M NaCl, on Fractogel EMD-SO_3^- at 0.85 M and on SP-Sepharose at 0.65 M NaCl. Thus, strongest interactions were taking place between Heparin-Sepharose and the protein, involving basic amino acids of bFGF such as arginine, lysine and histidine, and negatively charged groups at the heparin ligand [4], as displayed in Table 1. Both on the Fractogel EMD-SO_3^- and the SP-Sepharose the interactions rely on the negatively charged sulfonic acid groups. SDS-PAGE revealed that Heparin-Sepharose displayed highest selectivity towards bFGF compared to the other sorbents (Fig. 4) since host-specific proteins are barely visible on the silver-stained gel. The largest quantities of host-specific proteins were found with SP-Sepharose. On the Fractogel support contaminating proteins were also noticed, but in lower amounts (Fig. 4).

The concentration of bFGF in the eluted peak was highest on the SP-Sepharose, as demonstrated in Table 2. bFGF eluted later and in broader peaks on the other two sorbents which is indicative for the heterogeneity of the sorbents or kinetic effects during desorption. The differences of protein concentrations, as determined by Lowry and the scanner are probably due to interferences of Tris and NaCl with the Lowry method, which are most pronounced at elevated protein concentrations [28].

The heparin ligand is known from the literature to express a very heterogeneous composition [29]. Also the interacting ionic groups of heparin are of different origin (Table 1). Thus, the interaction strength of the sorbent varies and increasing salt concentrations will not lead to a sharp boundary during elution as commonly observed on ion exchangers but to gradual dissociation of the various ligand–solute complexes.

Fractogel EMD-SO_3 comprises polymeric ligands composed of acrylamide monomers with sulfonic acid functional groups. This ion exchanger behaves slightly different compared to common ion exchangers where each functional group is attached by a single spacer onto the matrix. Thus, this sorbent is also described as

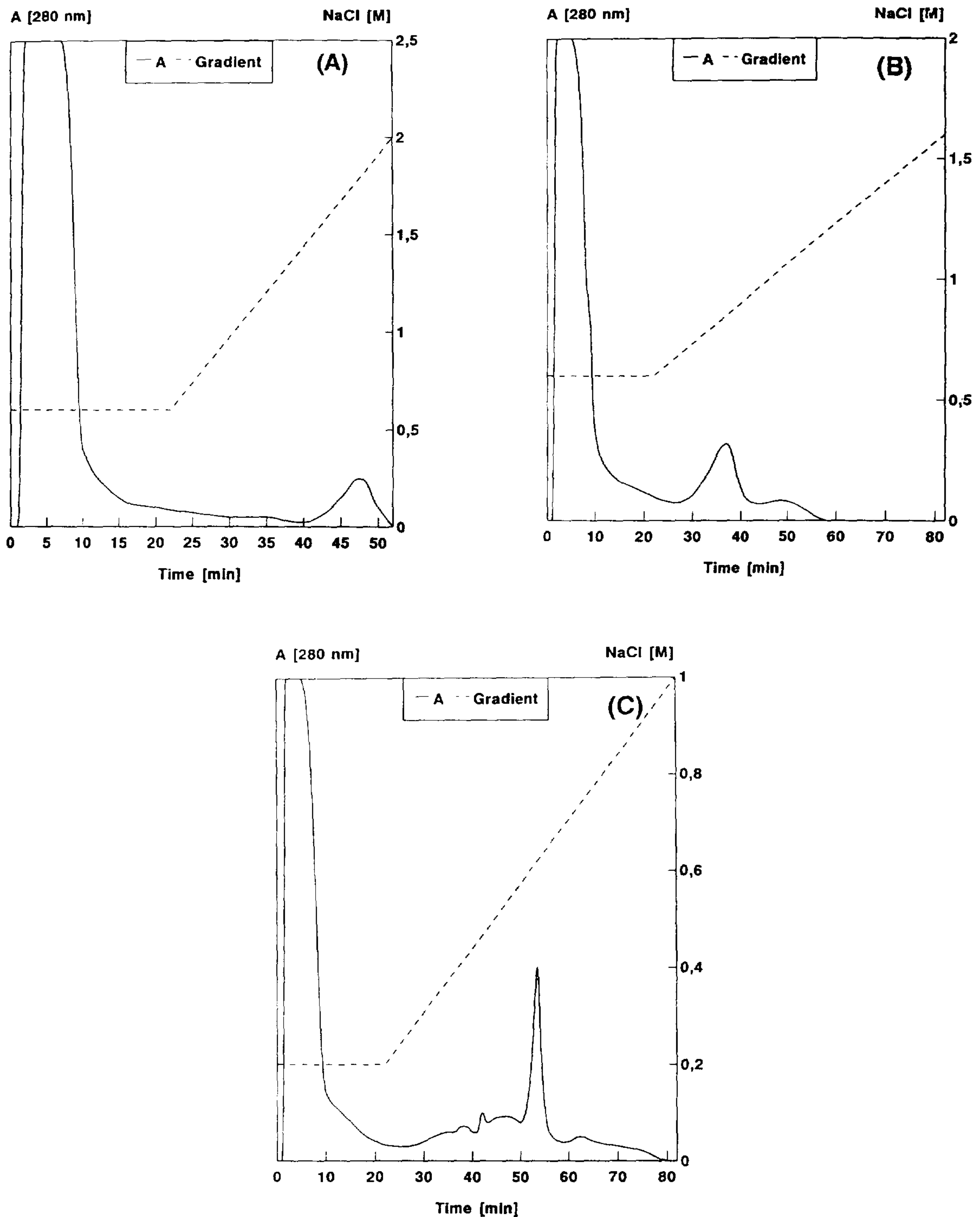

Fig. 3. Elution of bFGF on (A) Heparin-Sepharose, (B) Fractogel EMD-SO_3^- 650 (S), (C) SP-Sepharose. Column, 50 × 5 mm I.D.; flow-rate, 0.4 ml/min; temperature, 298 K; buffer, 20 m*M* Tris–HCl, pH 7.4; NaCl concentration during binding and gradient with increasing NaCl concentration as displayed.

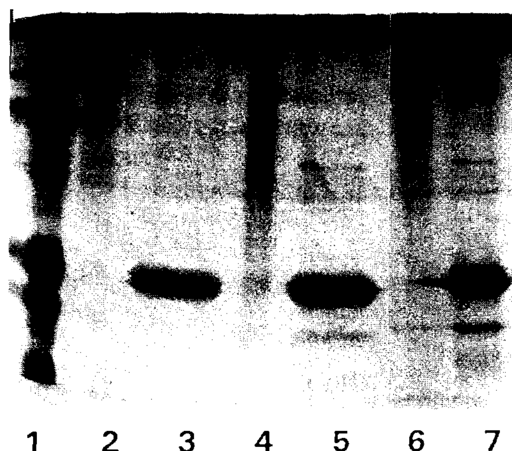

Fig. 4. SDS-PAGE of breakthrough and elution fractions during isolation of bFGF (18.4 kDa) using different sorbents (visualization by silver staining): Lane 1, protein standards (66 kDa, 45 kDa, 29 kDa, 18.4 kDa, 14.4 kDa, 6–8 kDa); lane 3, Heparin-Sepharose CL-6B; lane 5, Fractogel EMD-SO_3^- 650 (S); lane 7, SP-Sepharose (HP). Lanes 2, 4, and 6 represent the corresponding breakthrough fractions.

tentacle ion exchanger [30,31]. In case of bFGF it displays a stronger interaction than SP-Sepharose. This can be explained by the higher flexibility of polymeric chains leading to improved orientation of opposite charges at the protein and consequently to an increase of the contact area between bFGF and the polymeric ligand. This led to a higher selectivity for bFGF compared to traditional ion exchangers.

The Poros 20 HS sorbent, which is also a cation exchanger composed on a polymeric backbone, displayed adsorption and elution of bFGF as well. However, no peak of bFGF was observed at the salt concentration expected, but an increase in baseline absorption only. SDS-PAGE displayed bFGF at the position in the chromatogram assumed, however, in low concentrations and accommodating more contaminating proteins than the other functional sorbents. Since all Poros supports are based on a hydrophobic polystyrene matrix, 10% acetonitrile was added to the buffers, as is commonly recommended if complications occur with such matrices. However, the chromatogram did not change significantly. Also a decrease in the flow-rate from 5 ml/min, which is recommended, to 0.4 ml/min and the use of Tris–HCl instead of phosphate did not show any advantage. It is concluded that interaction of bFGF onto this sorbent is different from the other two ion exchangers under investigation. No explanation for this behaviour is found yet; these sorbents proved effective for other proteins under ion-exchange conditions [32,33]. Since bFGF was not purified by Poros 20 HS this sorbent was not further investigated.

3.1. *Stability of sorbents under CIP conditions*

The retention times on Heparin-Sepharose for bFGF decreased slightly after the first CIP. The difference could be overlooked at first glance. However, it decreased after each further CIP, whereas Fractogel EMD-SO_3^- and SP-Sepharose displayed almost identical results (Fig. 5). SDS-PAGE confirmed these results, indicating increasing contamination of the bFGF fraction with other proteins after CIP when Heparin-Sepharose was used, compared to a constant selectivity on Fractogel EMD-SO_3^- (Fig. 6).

The capacity for lysozyme decreased dramatically on the Heparin-Sepharose after the first CIP exhibiting only 50% or 40% of the original capacity with 0.2 or 1.0 *M* NaOH, respectively. A further decline of capacity was observed after

Table 2
Protein concentrations of eluted bFGF on different sorbents

Sorbent	Lowry c (mg/ml)	Scanner c (mg/ml)
Heparin-Sepharose CL-6B	0.17	0.17
Fractogel EMD-SO_3^- 650 (S)	0.18	0.15
SP-Sepharose (HP)	0.22	0.43

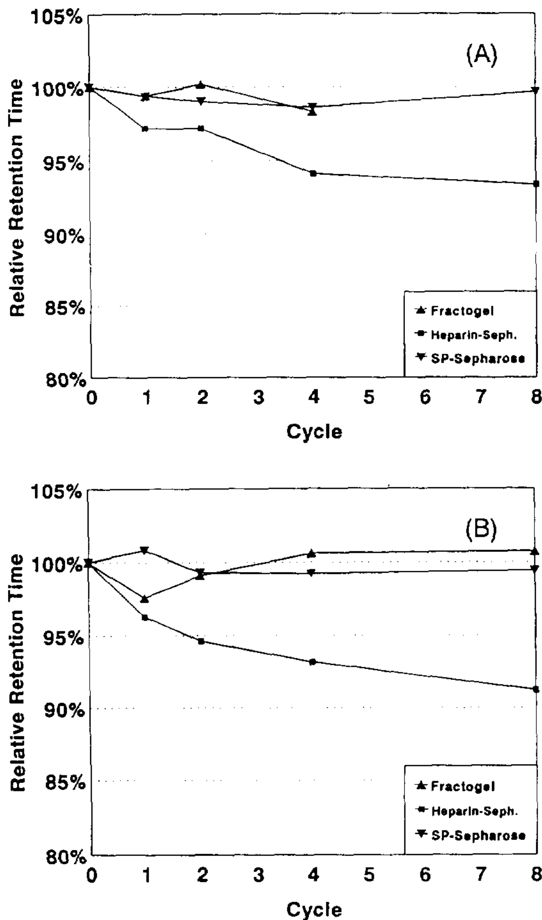

Fig. 5. Relative retention times of bFGF after CIP procedures; (A) 0.2 M NaOH, (B) 1.0 M NaOH.

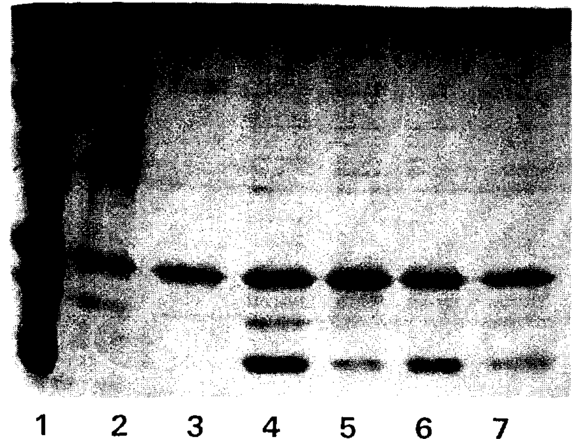

Fig. 6. Illustration of the purity of bFGF fractions on different sorbents after CIP by SDS-PAGE (bFGF, 18.4 kDa). Lane 1, protein standards as in Fig. 5; lanes 2, 3 and 4, Heparin-Sepharose CL-6B before CIP and after the fourth and eighth CIP; lanes 5, 6 and 7, Fractogel EMD-SO_3^- 650 (S) before CIP and after the fourth and eighth CIP.

each successive CIP (Fig. 7). In contrast, the capacity remained constant in the absence of CIP procedures. The capacities for lysozyme on Fractogel EMD-SO_3^- and SP-Sepharose were not affected by CIP procedures.

Thus, a heparin ligand is not suitable for CIP, since only the intact ligand displayed high selectivity towards bFGF. However, this selectivity was lost after treatment with aggressive chemicals, such as 0.2 or 1 M NaOH. Both Fractogel EMD-SO_3^- and SP-Sepharose did not display significant changes in capacity and selectivity after these treatments.

3.2. Purification of bFGF

Fractions obtained during elution of bFGF from Fractogel EMD-SO_3^- were applied directly onto Heparin-Sepharose without manipulation of eluted protein solutions. No breakthrough of contaminating proteins was observed on the second sorbent. Elution of bFGF occurred approximately at 1.64 M NaCl (data not shown). Beside bFGF no additional peak appeared on Heparin-Sepharose although the bFGF fraction contained small amounts of contaminating proteins after chromatography on the Fractogel support (see Fig. 4 for comparison). Since SDS-PAGE did not display contaminating proteins in the bFGF-containing fraction after purification on Heparin-Sepharose, they are probably irreversibly adsorbed onto this sorbent.

After dialysis against 20 mM phosphate buffer (pH 7.4) half of the bFGF solution was lyophilized and dissolved again in phosphate buffer in order to validate the product formulation. This procedure allowed to completely dissolve the protein after lyophilization. The final investiga-

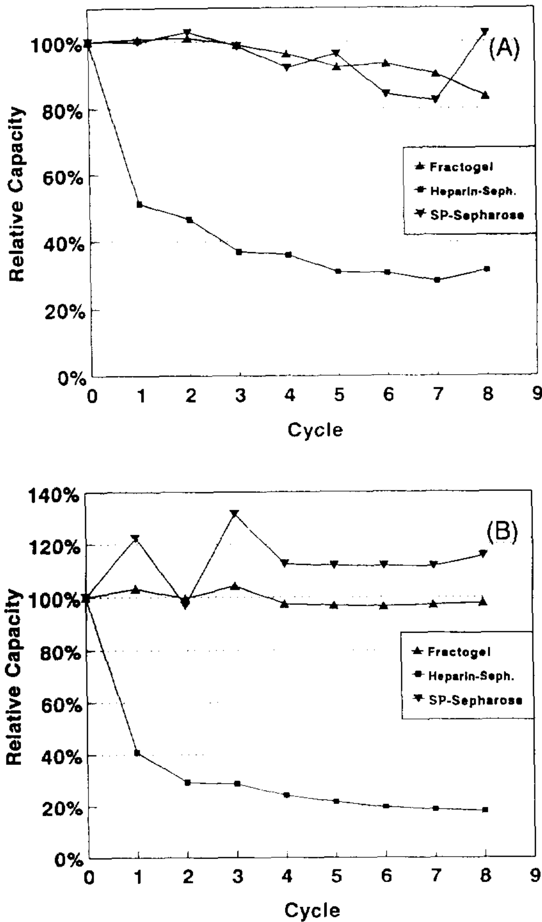

Fig. 7. Relative capacities for lysozyme as observed during CIP experiments with (A) 0.2 M NaOH and (B) 1.0 M NaOH.

tion of both solutions by CD spectroscopy revealed that also the three-dimensional conformation of bFGF was not affected by this treatment.

The same chromatographic procedure was then applied onto larger columns in order to yield higher quantities of the protein. The protein recovery after Heparin-Sepharose was rather low. Approximately 30% of the protein compared with the amount measured after the Fractogel column eluted from the heparin affinity sorbent. Even at prolonged treatment with 2 M salt no more bFGF eluted. SDS-PAGE analysis of fractions collected after the main peak did neither reveal any bFGF.

It was not possible to get thermodynamic data from the adsorption of bFGF onto the sorbents due to precipitation in 20 mM Tris + 0.2 M NaCl and protein concentrations around 1 mg/ml. Precipitation is known to occur with the recombinant bFGF due to formation of intermolecular cysteine bonds of Cys residues 78 and 96 following agglomeration of the protein [34,35]. In pituitary-derived bFGF, these amino acid residues are protected against oxidation by glutathione [36].

Agglomeration might also be the reason for the low recovery observed on Heparin-Sepharose. Maybe the locally high concentrations of bFGF at the sorbent and an optimal orientation of bFGF occurring during adsorption on the heparin ligand catalyzed this process.

3.3. Characterization of the sorbents by frontal chromatography of lysozyme

Fig. 8 displays the equilibrium isotherms obtained by adsorption of lysozyme onto the three

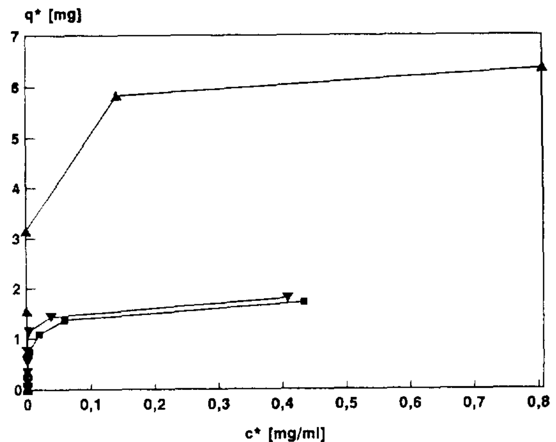

Fig. 8. Equilibrium isotherms for the adsorption of lysozyme onto three different sorbents in 20 mM Tris–HCl, pH 7.4, temperature 298 K: ■ Heparin-Sepharose CL-6B, ▲ Fractogel EMD-SO_3^- 650 (S), ▼ SP-Sepharose (HP).

Table 3
Capacity of different sorbents, q_m, for lysozyme and apparent dissociation constants, K_D, as determined by the Langmuir model, assuming single-site interaction of ligands and lysozyme

Sorbent	q_m (mg/ml)	K_D (M)
Heparin-Sepharose CL-6B	36.73	$6.35 \cdot 10^{-6}$
Fractogel EMD-SO_3^- 650 (S)	152.16	$2.16 \cdot 10^{-7}$
SP-Sepharose (HP)	40.83	$2.46 \cdot 10^{-6}$

sorbents. Obviously, strong interactions between all sorbents and lysozyme occur at low ionic strength, leading to a very steep incline of isotherms at low protein concentration. Isotherms close to such a rectangular shape are commonly observed employing ion exchangers as sorbents [37,38]. The constant incline of isotherms at elevated protein concentration is due to protein–protein interactions, as is common with lysozyme [39]. The largest capacity of the sorbents was observed with Fractogel EMD-SO_3^- (Table 3). Heparin-Sepharose and SP-Sepharose bear comparable capacities, however, less than 30% of the capacity of the Fractogel support. Apparent dissociation constants, as determined from double-reciprocal plots, assuming single-site adsorption, display the strong interaction at this salt concentration.

4. Conclusions

Heparin-Sepharose displayed the highest selectivity for bFGF of the sorbents under investigation. Contamination with host-specific proteins was almost absent after one chromatographic step of crude *E. coli* homogenate. However, bFGF recovery was found to be only about 30% with this sorbent which is probably caused by an irreversible binding process involving strong protein–protein binding of bFGF after adsorption.

Heparin-Sepharose was not stable under the CIP conditions employed, using 0.2 or 1 M NaOH. Both large changes in selectivity for bFGF after the first CIP and a large decrease in capacity for lysozyme was observed after the first CIP treatment. Thus, it cannot be employed for production if such conditions need to be applied. In a recently patented procedure the use of a Heparin sorbent was also circumvented [40].

From the other sorbents tested in this investigation Fractogel EMD-SO_3^- was the best alternative to Heparin-Sepharose although a slightly lower selectivity must be accepted. The higher selectivity of the Fractogel support compared to SP-Sepharose might be due to a better alignment of opposite charges of the flexible polymer chains and bFGF with the Fractogel sorbent compared to rather fixed charges at SP-Sepharose.

It also displayed at least three times the capacity for lysozyme of the other sorbents. Depending on the quality requirements for the final product, it might be necessary to include one further step with another sorbent. The direct application of a pooled fraction from Fractogel EMD-SO_3^- onto Heparin-Sepharose yielded a very pure product; however the recovery of bFGF after Heparin-Sepharose is low.

Agglomeration of the recombinant bFGF and formation of disulfide bonds between bFGF monomers is a general problem. This behaviour is object of current research in order to avoid precipitation in the final product.

Acknowledgements

Financial support was provided by the 'Hochschulnachwuchsförderungsprogramm' of the German federal ministry of education and research (HSP II).

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