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Journal of Chromatography A, 746 (1996) 17–24

JOURNAL OF CHROMATOGRAPHY A

Two-step chromatographic procedure for purification of basic fibroblast growth factor from recombinant *Escherichia coli* and characterization of the equilibrium parameters of adsorption

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Received 1 December 1995; revised 27 March 1996; accepted 28 March 1996

Abstract

A two-step chromatographic procedure for purification of basic fibroblast growth factor (bFGF) from high-cell-density cultures of recombinant *E. coli* is described. Heparin–Sepharose as a material which shows a high affinity to endothelial growth factors was used as sorbent for purification of bFGF from the soluble cell fraction. A one-step affinity chromatographic procedure resulted in very pure bFGF. However, this one-step affinity isolation of bFGF caused the loss of around 60% of the recombinant protein. A combination of ion-exchange chromatography with heparin–Sepharose affinity chromatography was favored for bFGF purification. A first cation-exchange chromatographic step resulted in a solution of bFGF with a purity of around 70%. The weak cation exchanger CM-Sepharose C50 was preferred in comparison to the strong cation exchanger S-Sepharose because of the higher recovery of bFGF. With the ion-exchange chromatographic step prior to the heparin–Sepharose affinity chromatography, the total yield of recovery of bFGF increased to 56% compared to 40% using the one-step purification procedure with heparin–Sepharose. To characterize the equilibrium parameters of adsorption, batch experiments for the calculation of maximum capacities and dissociation constants for CM-Sepharose C50 and heparin–Sepharose were carried out. The equilibrium experiments revealed that adsorption of bFGF to the ion-exchange sorbent followed single-site interaction according to the Langmuir model of adsorption. The adsorption of bFGF to heparin–Sepharose was described by a double Langmuir approach of two independent binding sites with different maximum capacities and dissociation constants. The purified bFGF showed a high biological activity and circular dichroic spectra of a proper folded molecule. The analysis of the N-terminal amino acid sequence revealed a mixture of two fractions of bFGF, which both are characterized by the cleavage of the first amino acid methionine. In addition, half of the bFGF molecules lacked the second amino acid alanine.

Keywords: *Escherichia coli*; Adsorption equilibria; Fibroblast growth factor; Peptides

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0021-9673/96/$15.00
PII S0021-9673(96)00286-5

1. Introduction

Basic fibroblast growth factor (bFGF) is a heparin-binding polypeptide that has been isolated from a variety of human and animal tissues and from normal as well as from tumor cell lines (e.g. Refs. [1–4]). This natural bFGF is only available in small quantities. For its increasing importance as a pharmaceutical protein, a high-cell-density cultivation method for the production of bFGF in recombinant *Escherichia coli* was developed [5]. In contrast to the low concentration of bFGF in natural sources, the concentration of the recombinant protein reached 4.9 g l^{-1} in high-cell-density cultures [5].

The purification procedure for isolation of bFGF from recombinant *E. coli* cultures is based on the observation of Shing et al. [6] that angiogenic factors have a strong affinity to heparin. The affinity of angiogenic factors to heparin has already been exploited to purify bFGF (e.g. Refs. [1,3,7,8]). Heparin is a highly sulfated glycosaminoglycan that has been shown to protect bFGF from acid and heat inactivation as well as from proteolytic degradation [9–11]. The crystal structures of heparin-derived oligosaccharides complexed with bFGF have been determined [12]. It has been shown that the high affinity binding site interacts with heparin through the amino acids Asn-28, Arg-121, Lys-126 and Gln-135 and the low affinity binding site through the amino acids Lys-27, Asn-102 and Lys-136 [12].

A critical point in the application of heparin–Sepharose for large-scale purification of bFGF is its instability under common cleaning-in-place conditions (CIP) [13]. Nevertheless, the material is resistant to ethanol, 0.1% Triton X, 6 *M* urea and 6 *M* guanidinium hydrochloride (Gnd–HCl). To avoid fast deterioration of heparin–Sepharose, a two-step procedure combining a cation-exchange chromatographic step with the affinity chromatography was employed. The high isoelectric point of bFGF (p*I*= 9.6) [2,14] offers a possibility for an effective removal of a large number of host cell derived impurities by cation exchange chromatography [13]. Final purification is achieved by affinity chromatography using heparin–Sepharose. The equilibrium parameters of adsorption of bFGF to heparin–Sepharose and the cation-exchange sorbent were determined in batch experiments and the maximum capacities and the dissociation constants were calculated according to the method of Langmuir [15]. Furthermore, the properties of the purified protein were investigated by spectroscopy, N-terminal sequence analysis and by its mitogenic activity.

2. Experimental

2.1. Production of bFGF

Temperature-induced production of bFGF (155-amino acid form) in high-cell-density cultures of recombinant *E. coli* TG1:pλFGFB was performed as described previously [5] except that product formation was induced at 25 g l^{-1} dry cell mass. The cells were harvested 16 h post-induction by rapid cooling on ice followed by centrifugation for 20 min at 6000 rpm at 4°C. The pellets were washed three times in TEN buffer (0.1 *M* Tris–HCl, pH 7, 0.01 *M* EDTA, 0.15 *M* NaCl) and resuspended in 50 m*M* sodium phosphate buffer (pH 7) to a final concentration of 40 g l^{-1} dry cell mass. Cells were lysed by passing the cell suspension 7 times through a French pressure cell (Manton Gaulin, France) at 700 bar. Cell debris and insoluble proteins were removed by centrifugation for 1 h at 10 000 rpm and 4°C. The supernatant fraction containing soluble bFGF was stored at −70°C.

2.2. Ion-exchange chromatography

After thawing, the supernatant fraction of the cell lysate was clarified by centrifugation at 13 000 rpm for 10 min. The clarified supernatant was dialyzed against 0.1 *M* sodium phosphate buffer (pH 6.3) and applied to a column packed with CM-Sepharose C50 or S-Sepharose (both sorbents from Pharmacia, Sweden). The sorbents were equilibrated at room temperature with 0.1 *M* sodium phosphate buffer (pH 6.3) using approximately 5 column volumes. The equipment used was a fast protein liquid chromatography (FPLC) system (Pharmacia, Sweden). The flow-rate was about 5 ml min^{-1}. Column dimensions were: diameter of 0.5 cm with a package volume of 1 ml or a diameter of 4.5 cm and a package volume of 100 ml for comparison of ion-exchange materials or for large-scale purifications,

respectively. Injection was carried out via an injection loop with a volume of maximum 10 ml or directly through the FPLC pump (100 ml). After loading the protein solution, the column was washed with 2–3 column volumes of 0.1 *M* sodium phosphate buffer (pH 6.3) until a stable baseline was reached. bFGF was eluted using a linear gradient of 0–1 *M* NaCl in 0.1 *M* sodium phosphate buffer (pH 6.3). For further investigations, the bFGF containing fraction was dialyzed overnight against 0.1 *M* sodium phosphate buffer (pH 6.3) at 4°C.

2.3. Heparin–Sepharose affinity chromatography

Clarified cell lysate or bFGF-containing fractions eluted from the ion-exchange sorbent were dialyzed against 0.1 *M* sodium phosphate buffer (pH 6.3) and bFGF was purified to homogeneity using HiTrap heparin–Sepharose-columns (Pharmacia) with a diameter of 0.5 cm and a volume of 1 ml. Equipment, equilibration and washing procedures as well as injection of protein solution were as described for the ion-exchange chromatography. bFGF was eluted by a linear gradient of 0–2 *M* NaCl in 0.1 *M* sodium phosphate buffer (pH 6.3) or after washing the column with 1 *M* NaCl in 0.1 *M* sodium phosphate buffer (pH 6.3) with a gradient of 1–2 *M* NaCl in 0.1 *M* sodium phosphate buffer (pH 6.3).

2.4. Adsorption isotherms

Isotherms for the adsorption of bFGF to CM-Sepharose C50 and heparin–Sepharose were determined in batch experiments. A constant volume of a suspension of ion-exchange sorbent or heparin–Sepharose in 0.1 *M* sodium phosphate buffer (pH 6.3) was added to a series of flasks containing a constant volume of bFGF at different concentrations in 0.1 *M* sodium phosphate buffer (pH 6.3). CM-Sepharose C50 and heparin–Sepharose applied for the batch experiments were exposed twice to column runs under standard conditions to avoid falsification of the results by a stronger adsorption of bFGF to new material. The flasks were incubated overnight on a rotary shaker (Eppendorf, Germany) at 20°C to establish equilibrium conditions. The chromatographic material was allowed to settle under gravity and equilibrium concentrations of bFGF in the supernatant were determined by UV-spectrophotometry at 280 nm. The extinction coefficient calculated from a calibration curve was 1.55 for 0.1% of bFGF. bFGF concentrations used for calibration were determined by amino acid analysis of hydrolyzed bFGF. The amount of bFGF adsorbed was then calculated by mass balance. Dissociation constants and maximum capacities of each sorbent were determined from the Langmuir isotherm (Eq. (1)),

$$a = \frac{a_m \cdot c}{K_D + c} \quad (1)$$

were K_D is the dissociation constant of the bFGF-sorbent complex [mg/ml] (bFGF: 1 *M* = 18 000 mg/ml), a is the adsorbed bFGF concentration [$mg_{bFGF}/ml_{sorbent}$], a_m the maximum capacity of the material [$mg_{bFGF}/ml_{sorbent}$] and c the equilibrium concentration of bFGF in the supernatant [mg/ml]. Data fitting was done by using the program Table Curve (Jandel Scientific, USA).

2.5. Gel electrophoresis

bFGF-concentrations in all fractions eluted from the columns were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [16]. Samples were mixed with sample buffer consisting of 7% (w/v) SDS, 35% (w/v) glycerol, 150 m*M* DTT (dithiothreitol), boiled for 10 min and electrophoresed immediately using precast gels (EXCEL-Gel 8–18, Pharmacia). Gels were stained with Coomassie Brilliant Blue R250. Quantification was done by densitometry (Hirschmann elscript 400, Germany) using bovine serum albumin (Sigma, Germany) for calibration. Coomassie-stained gels were additionally silver-stained (silver staining kit plusone, Pharmacia) to visualize minor contaminations in purified bFGF preparations.

2.6. Circular dichroic spectroscopy

Circular dichroic spectra were determined at room temperature on a Jasco J-600 spectropolarimeter. Measurements were carried out using a 0.1 ml cuvette for near and far UV spectra.

2.7. Amino acid composition and N-terminal sequencing

The amino acid composition of the purified protein was determined by its hydrolyzation (75 min in 6 *M* HCl at 160°C) and subsequent derivatisation of amino acids with phenylisothiocyanate using a hydrolyzer/derivatizer (420A/H, Applied Biosystems, USA). The amino acid derivatives were separated by reversed-phase HPLC and detected at 269 nm (Applied Biosystems). Automated N-terminal sequence analysis was performed on a protein sequencer (470A, Applied Biosystems) with on-line HPLC (12A, Applied Biosystems) via automated Edman degradation [17].

2.8. Bioassay

Mitogenic activity of purified bFGF was assayed on NIH 3T3 cells using the BrdU-Labeling-Kit (Boehringer Mannheim, Germany). Commercial available bFGF (Boehringer Mannheim) was used as standard. Stimulation, labeling, staining and quantification of the cells were performed according to the manufacturers instructions.

3. Results and discussion

3.1. Purification of bFGF

Recombinant bFGF with a concentration of 1.7 $g\,l^{-1}$ in clarified cell lysate was purified to homogeneity by a single chromatographic step using the affinity material heparin–Sepharose. Elution of a single peak of bFGF occurred at 1.2 *M* NaCl (Fig. 1). Purified bFGF was found to be homogeneous by gel electrophoresis and subsequent Coomassie staining (data not shown). In addition, no contaminating proteins were detected by N-terminal sequence analysis (data not shown). However, the sole application of heparin–Sepharose chromatography resulted in a very low recovery of bFGF. Only 40% of the applied bFGF was recovered from the column (Table 1). This low recovery may be due to irreversible binding to the sorbent and/or to interaction of bFGF with contaminating proteins. Since heparin-Sepharose is not resistant to common cleaning-in-place methods

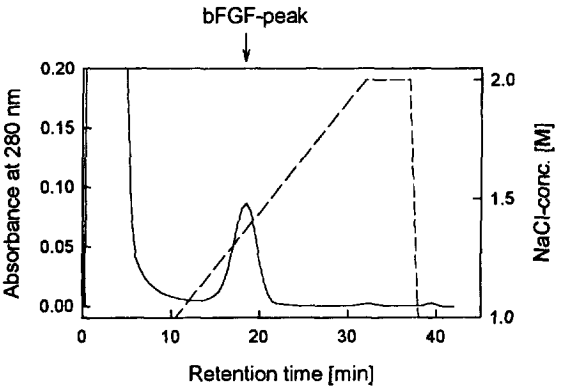

Fig. 1. Heparin–Sepharose chromatography of clarified cell lysate without prior ion-exchange chromatography. The clarified cell lysate was dialyzed against 0.1 *M* sodium phosphate buffer (pH 6.3) and 5 ml of the dialyzate with a concentration of 1.7 $g\,l^{-1}$ of bFGF were applied to a heparin–Sepharose column (1 ml). Elution was carried out using a 1 to 2 *M* NaCl gradient. Elution of bFGF occurred at 1.2 *M* NaCl.

[13] a purification procedure including an additional application of a cation exchanger for prior removal of contaminating proteins was envisaged. A weak and a strong cation exchanger, CM-Sepharose C50 and S-Sepharose, respectively, were compared with regard to purity and recovery of applied bFGF. From the eluted material, bFGF containing fractions were applied to the heparin–Sepharose column. The results of the different purification steps are summarized in Table 1. The strong and the weak cation exchanger did not show any significant difference with respect to purity and recovery of bFGF. The application of both materials caused the elimination of the majority of host cell contaminating proteins (Fig. 2). The recovery of bFGF was around 5% higher using CM-Sepharose C50 as the weak cation exchanger compared to S-Sepharose as the strong cation exchanger. However, more host cell protein impurities were removed using the strong cation exchanger S-Sepharose. Taking into account that the ion-exchange step is followed by affinity chromatography, further investigations were carried out using CM-Sepharose C50 for its better recovery of the recombinant protein. A typical chromatogram for the elution of bFGF from CM-Sepharose C50 is shown in Fig. 3. With the application of a cation exchange chromatographic step prior to heparin–Sepharose chromatography, the recovery of bFGF using

Table 1
Comparison of different chromatographic sorbents used for purification of bFGF

Material	CM-Sepharose C50	S-Sepharose	Heparin–Sepharose	
Purity of bFGF[a]	70%	75%	≈99%	
Max. capacity[b]	21.5 mg ml^{-1}	–	>54 mg ml^{-1}	
Dissociation constant K_D^b	$1.2 \cdot 10^{-6}$ *M*	–	$1.5 \cdot 10^{-7}$ *M* $1.3 \cdot 10^{-4}$ *M*	
Recovery of bFGF[a]	70%	65%	80% with IE[c]	40% without IE[c]
CIP-resistance[d]	2 *M* NaCl 1 *M* NaOH 0.1–0.5% Triton X 0.1 *M* Acetic acid 70% Ethanol 30% Isopropanol	2 *M* NaCl 1 *M* NaOH 0.1–0.5% Triton X 0.1 *M* Acetic acid 70% Ethanol 30% Isopropanol	0.1% Triton X 6 *M* Urea 6 *M* Gnd–HCl	

[a] Values determined from Coomassie-stained SDS gels by densitometry.
[b] Values determined from batch experiments.
[c] IE Ion-exchange sorbent.
[d] Information from manufacturer (Pharmacia, Sweden).

heparin–Sepharose chromatography increased to 80%. The total yield of recovery using the combined application of CM-Sepharose C50 and heparin–Sepharose was calculated to be about 56%. A maximum purification factor was not determined, because the maximum capacity of the above materials with respect to bFGF were not reached and, therefore, the eluted bFGF was not as concentrated as possible. With increasing bFGF-concentrations in the eluted fraction precipitation of the protein was observed.

When heparin–Sepharose chromatography was

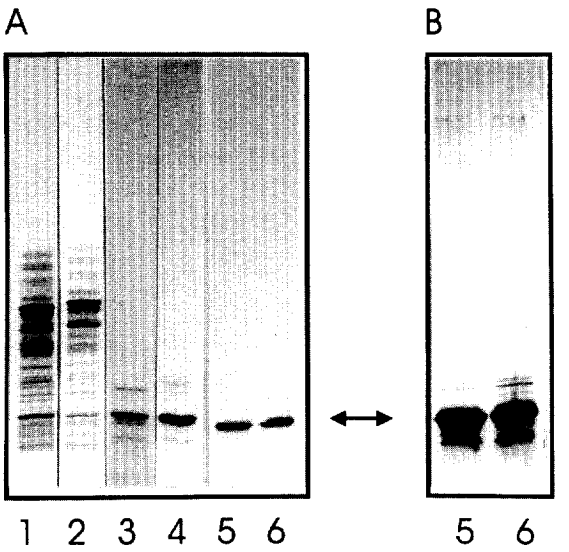

Fig. 2. (A) Coomassie Brilliant Blue and (B) silver stained SDS gel of different bFGF-containing fractions. Lane 1: Total cell lysate; Lane 2: Soluble cell lysate; Lane 3: bFGF-fraction eluted from the CM-Sepharose C50 column; Lane 4: bFGF-fraction eluted from the S-Sepharose column, Lanes 5 and 6: bFGF-fractions eluted from the heparin–Sepharose column with prior cation-exchange chromatography corresponding to peaks 2 and 1, respectively. The arrow indicates the position of bFGF.

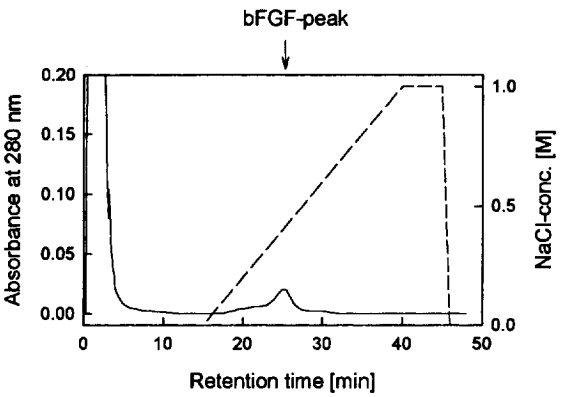

Fig. 3. CM-Sepharose C50 chromatography of clarified cell lysate. The clarified cell lysate was dialyzed against 0.1 *M* sodium phosphate buffer (pH 6.3) and 2 ml of the dialyzate with a concentration of 1.7 $g l^{-1}$ of bFGF were applied to a CM-Sepharose C50 column (1 ml). Elution was carried out using a 0 to 1 *M* NaCl gradient. Elution of bFGF occurred at 0.4 *M* NaCl.

applied to the bFGF-containing fraction collected after ion exchange chromatography, one major and one minor peak corresponding to bFGF were eluted from the heparin–Sepharose column at NaCl concentrations of 1.5 *M* and 1.9–2 *M*, respectively (Fig. 4). SDS-PAGE analysis of fractions corresponding to both peaks revealed in each case very pure bFGF without any difference in the electrophoretic mobility, minor contaminations were only visualized by overstaining using silver staining techniques (Fig. 2). Structural analysis of bFGF corresponding to the two different peaks by circular dichroic spectroscopy (far and near UV) did not reveal any significant difference (data not shown). These results correspond to previous observations reported by Ke et al. [18] and Iwane et al. [19] who also found one major and one minor peak containing bFGF after elution from heparin–Sepharose columns. In addition, it was proven that bFGF preparations corresponding to the different peaks were indistinguishable with respect to amino acid composition and purity [19].

3.2. Characterization of the equilibrium parameters of adsorption

The experimental results of adsorbed bFGF to CM-Sepharose C50 at equilibrium were plotted against the free bFGF concentration in solution and

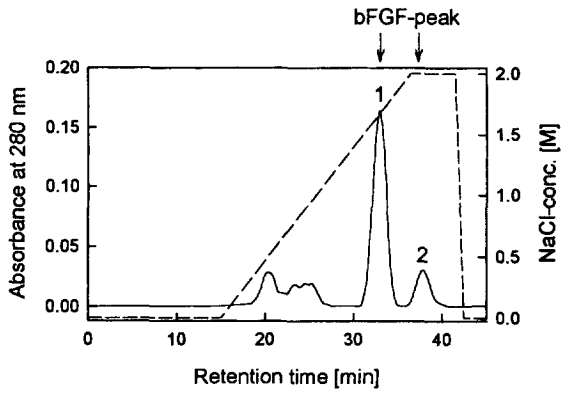

Fig. 4. Heparin–Sepharose chromatography of bFGF-containing fractions collected after ion-exchange chromatography. The bFGF-containing fractions were dialyzed against 0.1 *M* sodium phosphate buffer (pH 6.3) and applied to a heparin–Sepharose column (1 ml). Elution was carried out using a 0 to 2 *M* NaCl gradient. Elution of bFGF occurred at 1.5 *M* NaCl (peak 1) and 1.9–2 *M* NaCl (peak 2).

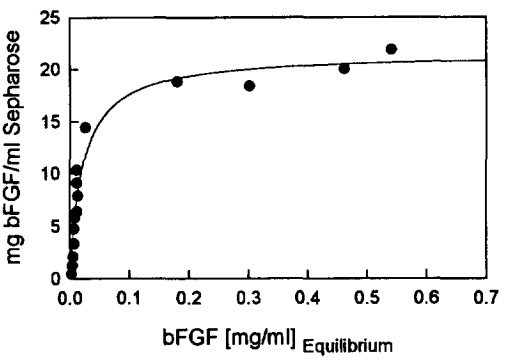

Fig. 5. Isotherm of the adsorption of bFGF to CM-Sepharose C50 in 0.1 *M* sodium phosphate buffer (pH 6.3) at 20°C. The experimental data (●) are plotted as bound bFGF [$mg_{bFGF}\ ml^{-1}_{CM\text{-}Sepharose\ C50}$] versus free bFGF [mg ml^{-1}]. The solid line was determined by data fitting assuming the simple Langmuir model of adsorption as described in Section 2.4

data fitting was done assuming a simple Langmuir model of adsorption (Fig. 5). The results revealed that binding of bFGF to CM-Sepharose C50 follows single-site interaction of bFGF and sorbent corresponding to the Langmuir model of adsorption. The apparent dissociation constant K_D and the maximum capacity of the material for bFGF a_m were calculated to be $1.24 \cdot 10^{-6}$ *M* and 21.5 $mg_{bFGF}\ ml^{-1}_{CM\text{-}Sepharose\ C50}$, respectively.

The adsorption of bFGF to heparin–Sepharose did not follow the Langmuir model of single-site interaction (data not shown). By rearrangement of the Langmuir equation (Eq. (1)) to the linearized form of Eq. (2),

$$\frac{a}{c} = -\frac{1}{K_D} \cdot a + \frac{a_m}{K_D} \qquad (2)$$

a Scatchard analysis of the binding of bFGF to heparin–Sepharose was carried out (Fig. 6). The shape of this curve matches with a two binding sites system. The dissociation constants K_{D1} and K_{D2} corresponding to the dissociation constants of the first and the second binding sites of bFGF to heparin–Sepharose, respectively, and the maximum capacities a_{m1} and a_{m2} corresponding to the maximum capacities of the first and the second binding sites of bFGF to heparin–Sepharose, respectively, were determined from the slopes and the *y*-intercepts of the Scatchard plot. Resulting values were used as

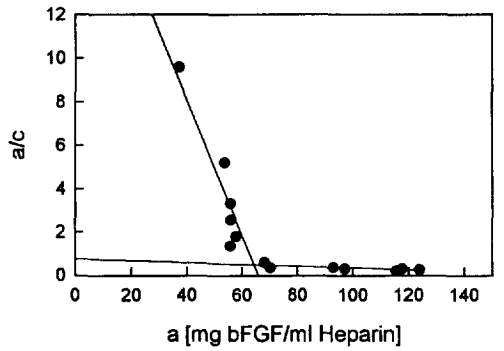

Fig. 6. Scatchard analysis of the adsorption of bFGF to heparin–Sepharose in 0.1 *M* sodium phosphate buffer (pH 6.3) at 20°C. The experimental data (●) are plotted as the ratio of bound [$mg_{bFGF}\ ml^{-1}_{heparin-Sepharose}$] to free bFGF [$mg\ ml^{-1}$] versus the free bFGF [$mg\ ml^{-1}$]. Solid lines were determined by regression analysis assuming two-binding sites interaction of bFGF with the sorbent.

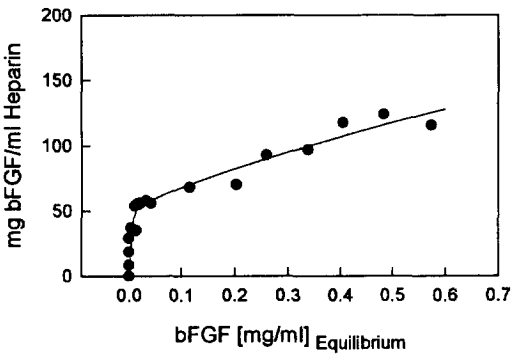

Fig. 7. Isotherm of the adsorption of bFGF to heparin–Sepharose in 0.1 *M* sodium phosphate buffer (pH 6.3) at 20°C. The experimental data (●) are plotted as bound bFGF [$mg_{bFGF}\ ml^{-1}_{heparin-Sepharose}$] versus free bFGF [$mg\ ml^{-1}$]. The solid line was determined by data fitting assuming the double Langmuir model of adsorption as described in Section 3.2

initial values to fit the experimental data according a two-sites interaction of binding of bFGF to heparin–Sepharose using a double Langmuir approach (Eq. (3)).

$$a = \frac{a_{m1} \cdot c}{K_{D1} + c} + \frac{a_{m2} \cdot c}{K_{D2} + c} \quad (3)$$

The final values for the apparent dissociation constant K_{D1} and the maximum capacity of the sorbent for bFGF a_{m1} of the first binding site were calculated to be $1.46 \cdot 10^{-7}$ *M* and 55 $mg_{bFGF}\ ml^{-1}_{heparin-Sepharose}$, respectively. The final values for the apparent dissociation constant K_{D2} and the maximum capacity of the sorbent for bFGF a_{m2} of the second binding site were determined to be $1.3 \cdot 10^{-4}$ *M* and 361.3 $mg_{bFGF}\ ml^{-1}_{heparin-Sepharose}$, respectively. Thus, the experimental data of adsorption of bFGF to heparin–Sepharose are described by a double Langmuir approach of two-sites interaction (Fig. 7).

The application of the Langmuir model for analysis of protein adsorption to ion exchange as well as to affinity sorbents, especially in the region of strong binding, has been criticized, because there is experimental evidence that proteins do not bind at individual and independent binding sites, but that multivalent binding involving several functional groups on both the protein and the adsorber occurs [20]. Although the single and the double Langmuir approach may not have direct physical significance, it conforms to the experimental results of binding of bFGF to the cation exchange sorbent and to heparin–Sepharose, respectively. The dissociation constant corresponding to the high affinity binding site is similar to the one reported by Faham et al. [12] for binding of bFGF to a heparin-derived hexasaccharide ($K_D = 1.01 \cdot 10^{-7}$ *M*). Nevertheless, the conclusion that adsorption of bFGF to heparin–Sepharose follows a double Langmuir approach is also compatible with dimer formation at higher concentrations of bFGF. For example, it has been shown that heparin-derived oligosaccharides can stabilize bFGF self-association [21].

3.3. Characterization of purified bFGF

Circular dichroic spectra obtained from pooled fractions of pure bFGF (0.3 $mg\ ml^{-1}$) in 0.1 *M* sodium phosphate buffer (pH 7) did not reveal any significant difference compared to circular dichroic spectra of recombinant bFGF shown by Arakawa et al. [22], (data not shown).

The purified recombinant bFGF was also tested for its mitogenic activity on 3T3 cells. For comparison, commercial available bFGF (Boehringer Mannheim,) was used as standard. The results revealed that the mitogenic activity of bFGF prior and after purification did not differ from the mitogenic activity of the commercial available product (data not shown) dem-

onstrating that the purification procedure did not cause inactivation of the recombinant protein.

N-terminal sequence analysis demonstrated complete removal of the N-terminal methionine. However, half of the recombinant purified bFGF exhibited additional removal of the following N-terminal alanine. This result corresponds to previous observations of N-terminal heterogeneity of bFGF produced by different recombinant hosts such as *E. coli* [18,23–25] or *S. cerevisiae* [24]. The N-terminal heterogeneity is caused by the extreme susceptibility of the N-terminus of bFGF to proteolysis [26]. It has been shown that natural bFGF exhibits N-terminal heterogeneity and that cleavage of bFGF to lower molecular weight forms does not affect the biological activity of the protein [4,26]. If required, a homogeneous N-terminus can be generated by specific proteolysis of the larger microheterogeneous forms to the 146-amino acid form of bFGF [25].

Acknowledgments

The authors would like to thank C. Ragge for analyzing the mitogenic activity of bFGF and M. Kiess for determination of the amino acid composition and N-terminal sequence of bFGF. We gratefully acknowledge continuous support by W.-D. Deckwer.

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