Affinity chromatography of fibroblast growth factors on coated silica supports grafted with heparin

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ABSTRACT

Dextran-coated silica beads are excellent supports for affinity chromatography of proteins. They can be easily grafted using conventional coupling methods with different active ligands, such as heparin. Fibroblast growth factors develop specific interactions with heparin through well-defined amino acids sequences. The heparin dextran coated silica phases exhibit an affinity for these growth factors. Under our experimental conditions, the basic form can be absorbed on the solid support at a moderate salt concentration (0.5 M sodium chloride) and can be selectively desorbed by increasing the ionic strength of the eluent. The purification performances of such phases are compared to those obtained on the heparin grafted soft gels. Because of their mechanical properties, the dextran-coated silica supports were also used in high-performance affinity chromatography to purify fibroblast growth factors from a bovine brain crude extract.

INTRODUCTION

Growth factors, known as fibroblast growth factors (FGF) [1] or heparin binding growth factors [2], are implicated in the growth of different types of cells, particularly fibroblasts and endothelial cells [3,4]. Among their biological activities, angiogenic and wound-healing properties are known and have been studied in order to use fibroblast growth factors in therapy [4,5].

Fibroblast growth factors are able to develop specific interactions with heparin through well defined amino acid sequences and the two forms of FGF, acidic FGF (aFGF) and basic FGF (bFGF), are generally purified by affinity chromatography on heparin-grafted soft gels.

In a previous paper [6], we demonstrated that polystyrene resins can be functionalized in order to obtain a specific affinity for fibroblast growth factors. These synthetic supports can also be used for the purification of FGF by affinity chromatography [7].

However, coated silica supports are excellent stationary phases for the high-performance affinity chromatography (HPAC) of proteins [8]. The passivation of native silica can be achieved by covering the inorganic surface with a layer of a hydrophilic polymer (agarose) substituted with a calculated amount of positively charged diethylaminoethyl (DEAE) functions. Thus the non-specific adsorption of

proteins is minimized. Because of their polymeric coating, these supports can easily be grafted with functional derivatives, in particular with heparin, using conventional coupling methods. These affinity phases possess the mechanical properties of the starting silica and the hydrophilicity of the coating polysaccharide [9] and they can be used with low-pressure or high-performance liquid chromatographic (HPLC) elution conditions in order to separate or purify fibroblast growth factors. The chromatographic performances of these new affinity supports can be compared with the results observed under the same elution conditions on the conventional heparin–Sepharose affinity phase [10].

EXPERIMENTAL

Growth factors

Crude extracts and radiolabelled fibroblast growth factors were obtained as described previously [11]. Briefly, crude extract is prepared from a bovine brain by ammonium sulphate fractionation (the active fraction was found to be between 20 and 60%) at 4°C, followed by dialysis against acetic acid and phosphate buffer solutions. The sample is then centrifuged (20 000 g, 30 min, 4°C) and the ionic strength of the supernatant containing aFGF and bFGF is adjusted to 0.65 M NaCl. Acidic and basic fibroblast growth factors are purified from bovine brain crude extract by affinity chromatography on heparin–Sepharose support, and are radiolabelled with 125-iodine using the chloramine T method [6].

All reagents were of analytical-reagent grade and all solutions and buffers were prepared with doubly distilled water, which was degassed and filtered through a 0.22-μm membrane.

Heparin–silica supports

The silica-based (Hep–SiA) supports are prepared in two stages as described previously [9]. Agarose Indubiose A37 HAA, kindly provided by IBF Biotechnics (Villeneuve la Garenne, France), are modified by a controlled substitution with 2-diethylaminoethyl hydrochloride (Janssen Chemica, Pantin, France). First, silica beads X015 M (porosity 1250 Å, particle size 40–100 μm) from IBF Biotechnics are impregnated with a concentrated solution of DEAE-polysaccharide and the polymer coating is cross-linked in order to prevent leakage of the polymeric layer and to obtain a polymeric coverage of 4.5 g per 100 g of dry support. In the second step, heparin (101 I.U./mg), supplied by Institut Choay (Paris, France), is coupled using 1,1′-carbonyldiimidazole (CDI) as activating agent [10]. The yield of the coupling is 68%, corresponding to 34 mg of heparin per 1 g of support.

Low-pressure affinity chromatography

Two columns (30 × 11.4 mm I.D.) are packed, one with 4 ml of heparin–Sepharose (Pharmacia) and the other with 4 ml of heparin–dextran-coated silica phase in phosphate buffer solution (PBS) adjusted to 0.5 M NaCl (pH 7.4). This salt concentration (0.5 M NaCl) was chosen because aFGF could be retained by the support with a lower salt concentration and other proteins could be desorbed simultaneously by the salt gradient. Similarly, it is impossible to obtain pure aFGF by affinity chromatography on the heparin–Sepharose support if the initial salt concen-

tration of the eluent is lower than 0.5 M. A 5-ml sample of crude bovine brain extract mixed with radiolabelled FGFs (10^6 cpm) is injected into the columns. The non-adsorbed compounds are eliminated by washing with 150 ml of 10 mM PBS–3 mM KCl–0.5 M NaCl, pH 7.4). Next, the adsorbed proteins are desorbed using a salt gradient from 0.5 to 2.5 M NaCl (150 ml) at a flow-rate of 24 ml/h at room temperature. The absorbance at 280 nm and the ionic strength of the eluent are measured using an A111 UV detector (Gilson, Villiers le Bel, France) and an ion and pH detector (IBF Biotechnics), respectively. The amount of eluted radiolabeled fibroblast growth factor is determined using a gamma counter.

High-performance affinity chromatography

The HPLC apparatus is a Merck–Hitachi 655 A-12 system from Labs. Merck-Clevenot (Nogent sur Marne, France). A stainless-steel column (70 × 7 mm I.D.) is packed using a slurry method with 2.6 ml of heparin–silica phase in phosphate buffer solution (pH 7.4) adjusted to 0.5 M NaCl. A 5-ml aliquot of crude bovine brain extract is injected onto the column and the non-adsorbed compounds are eluted with the initial buffer (10 mM PBS–0.5 M NaCl, pH 7.4) at a flow-rate of 1.0 ml/min. The adsorbed proteins are then eluted by a linear salt gradient from 0.5 to 2 M NaCl in 0.01 M PBS (pH 7.4). The elution is followed using a variable-wavelength UV monitor at 280 nm. The eluted fractions are collected and characterized.

Characterization of eluted fractions

The collected fractions from low-pressure or HPLAC runs are dialysed, concentrated and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (20% acrylamide) according to Laemmli [12], using a Pharmacia Phast System (Pharmacia LKB, Uppsala, Sweden). Proteins are revealed by silver staining [13].

Fractions are also tested for mitogenic activity on Chinese hamster lung fibroblasts (CCL39, American Type Culture Collection) by reinitiation of DNA synthesis as described previously [6].

RESULTS AND DISCUSSION

The heparin–silica supports interact with fibroblast growth factors in solution. The mixture of crude extract and radiolabelled aFGF and bFGF is eluted on heparin–silica (Fig. 1) and heparin–Sepharose (Fig. 2) in a low-pressure affinity chromatography procedure. The elution profiles of radiolabelled FGFs indicate that they are mainly retained by the solid phases at lower ionic strength and desorbed by the salt gradient.

The rates of radiolabelled FGFs reversibly adsorbed on the two supports are presented in Table I. The comparison with heparin–Sepharose support shows that, under our experimental conditions, bFGF exhibits a similar behaviour on both supports (Table I). However, under the same conditions aFGF is not well retained on the silica phases. It is also important to note that both fibroblast growth factors, when they are adsorbed on the affinity phases, are more easily desorbed from the heparin–silica supports by the salt gradient, probably indicating a smaller contribution of ionic interactions in the affinity mechanism on this support. A similar effect has
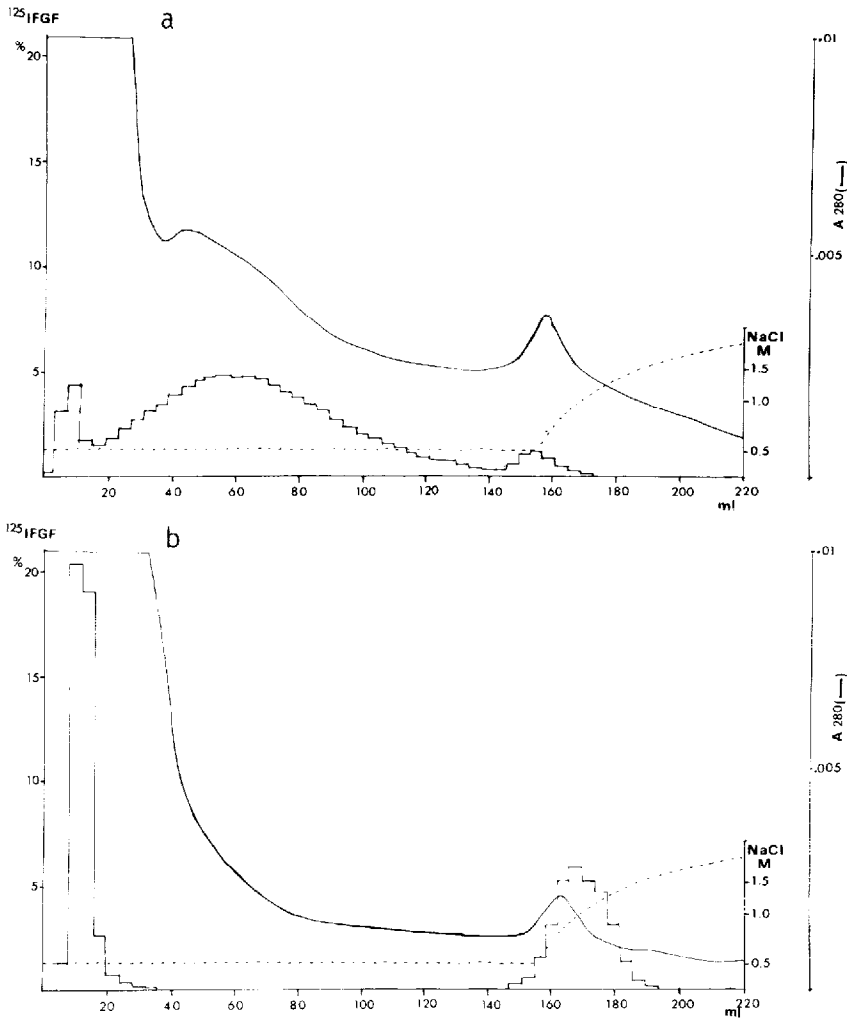
Fig. 1. Low-pressure chromatographic assay of bovine brain crude extract (5 ml) containing radiolabelled aFGF (a) or bFGF (b) on heparin agarose-coated silica support.

been described for bFGF eluted from heparin affinity HPLC supports [14]. This weaker affinity is probably the reason for the low adsorption of aFGF under our initial eluting conditions (0.01 M PBS 0.5 M NaCl). Nevertheless, a complete comparison between the two types of affinity supports is difficult because of the non-equivalence of the biological activities of the grafted heparin. First, heparin fixed on commercial Sepharose support is presumably different from the heparin grafted on our silica phases: the molecular mass, the specific anticoagulant activity and the distribution of sugar groups along the network are probably different. Moreover, the coupling of the polysaccharide also depends on the grafting reactions. The macromolecular chain can
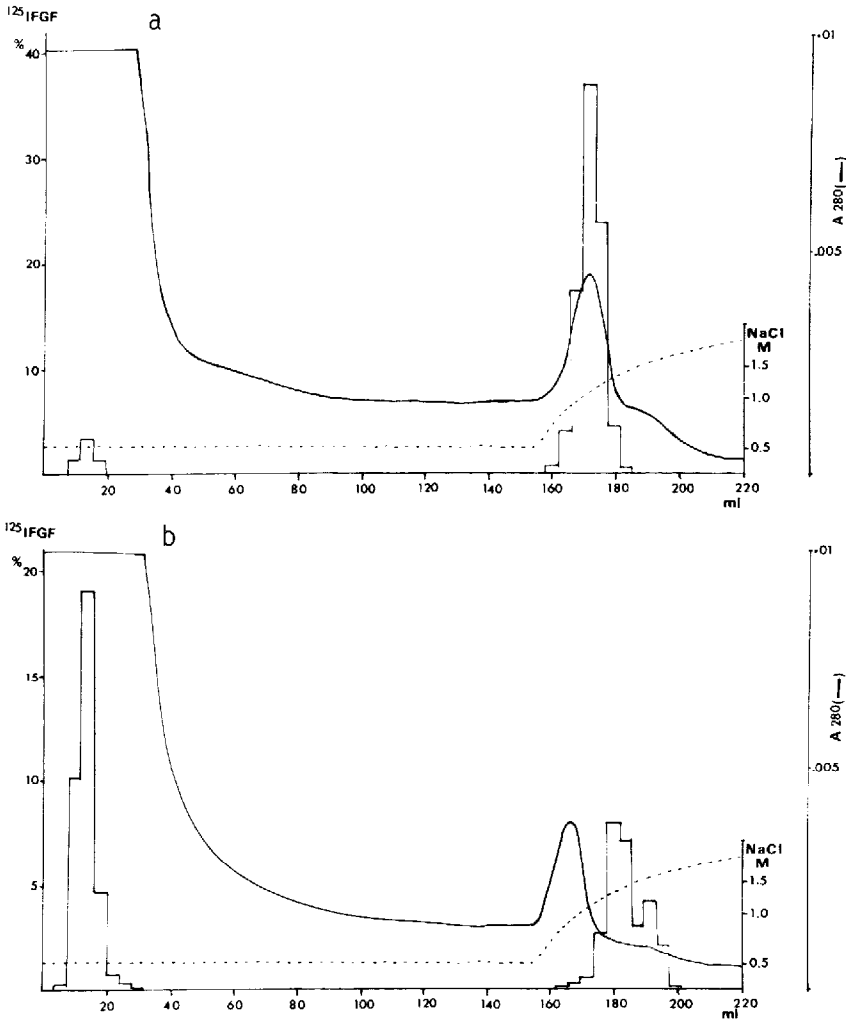
Fig. 2. Low-pressure chromatographic assay of bovine brain crude extract (5 ml) containing radiolabeled aFGF (a) or bFGF (b) on heparin Sepharose support.

be attached to the Sepharose or agarose structure in different ways with different covalent bonds and consequently the active sequence of heparin is not necessary accessible to aFGF. It is reasonable to assume that the affinity for aFGF could be reinforced by grafting more active heparin fractions.

The HPLAC elution profile of bovine brain crude extract on silica-based resins is presented in Fig. 3. Under our experimental conditions, the proteins are mainly eluted in the washing buffer. When the ionic strength of the eluent is increased, a small amount of proteins (15 μg) is desorbed in a large peak (fraction F). The presence of aFGF and bFGF in this eluted peak is revealed by SDS-PAGE. It should be noted that many other proteins are simultaneously eluted and they can disturb the adsorption

TABLE I

ADSORPTION CONDITIONS OF GROWTH FACTORS ON ACTIVE SUPPORTS

Resin[a]	FGF	NA (%)[b]	RA (%)[b]	NaCl (M)[c]
Hep–Seph	aFGF	1	89	1.0
Hep–Seph	bFGF	36	29	1.5
Hep–SiA	aFGF	86	9	0.8
Hep–SiA	bFGF	46	31	1.2

[a] Hep–Seph = heparin–Sepharose support; Hep–SiA = heparin–agarose-coated silica support.
[b] NA (%) = percentage of FGF washed under the initial conditions; RA (%) = percentage of FGF retained and desorbed by the gradient.
[c] NaCl concentration of the gradient corresponding to the maximum of the eluted peak.

process. However, these fractions also stimulate the cellular growth of fibroblast CCL39 (Fig. 4). The stimulation unit of these fractions, which is the concentration needed to obtain 50% of the maximum stimulation, is weaker (2 ng/ml) than those observed under the same conditions with pure aFGF (10 ng/l) and bFGF (3.8 ng/ml). As observed, the eluted fractions obtained from the crude extract are more effective; this could be explained by a smaller deactivation of fibroblast growth factors during the chromatography owing to a faster separation process. This purification procedure is efficient for bFGF but the initial conditions of elution have to be optimized with aFGF. It is also possible to increase the affinity of the heparin-grafted support for aFGF by modifying the coupling method. Moreover, the mechanical properties of the silica phases allow the use of higher flow-rate leading to faster purification.
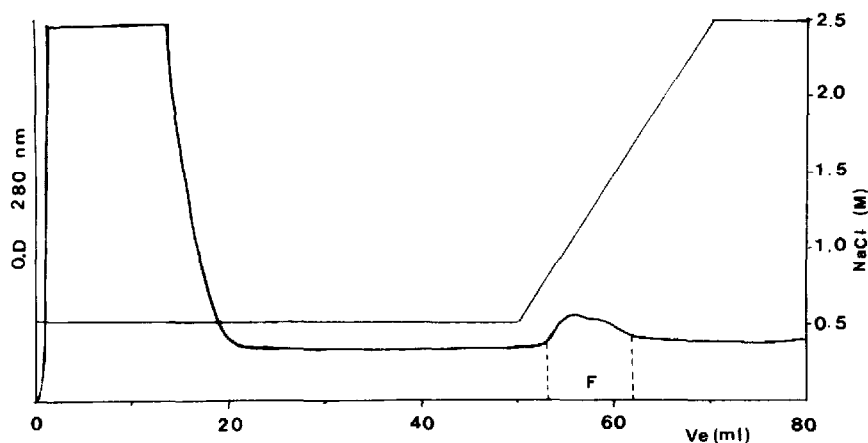

Fig. 3. HPLC of crude bovine brain extract (9 ml) on heparin–agarose-coated silica support.
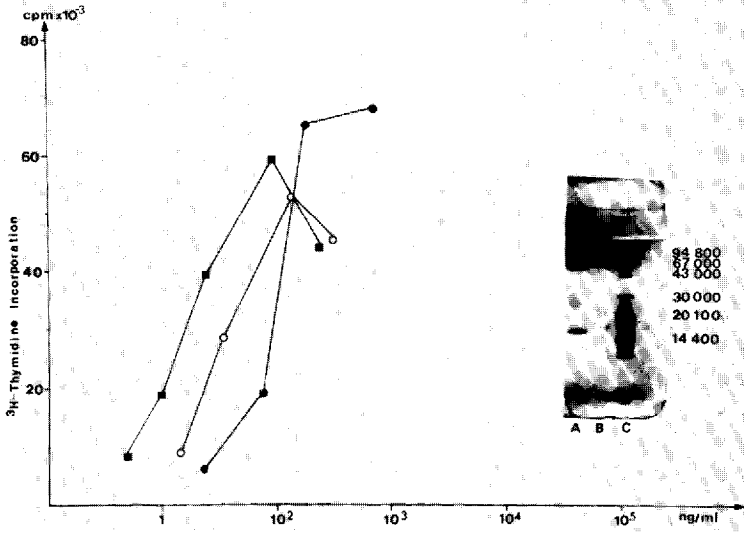
Fig. 4. Effect of (●) aFGF, (○) bFGF and (■) eluted fraction F on the incorporation of [³H]thymidine on CCL39 fibroblast. Inset: SDS-PAGE of (B) bFGF eluted from heparin–Sepharose and of (A) fraction F eluted from heparin–dextran-coated silica; (C) molecular mass markers.

CONCLUSION

The affinity of fibroblast growth factors for heparin is used in chromatography for the purification of these factors. These growth factors are generally purified on heparin–Sepharose support under low-pressure elution conditions. The purification of fibroblast growth factors can be also achieved on agarose-coated silica supports grafted with heparin directly from a bovine brain crude extract. The stimulating effect of eluted fractions on fibroblast growth shows that an important amount of fibroblast growth factor is retained and desorbed from the affinity stationary phase. In fact, the elution of radiolabelled pure FGF demonstrates that on both supports the basic form (bFGF) is strongly adsorbed at low ionic strength and selectively desorbed by the salt gradient. In contrast, aFGF seems to exhibit a weaker affinity for the heparin–agarose coated silica support. Under our initial elution conditions, this growth factor is mainly unretained and eluted by the washing eluent. In order to improve the affinity of aFGF for the heparin–agarose-coated silica support with minimum adsorption of other proteins, the initial conditions of the separation have to be optimized. However, the mechanical properties of such silica-based affinity phases are excellent, they allow fast separations and the purification procedure of fibroblast growth factors can easily be scaled up.

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