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High-performance affinity chromatography of basic fibroblast growth factor on polystyrene sulphonate resins modified with serine

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ABSTRACT

Basic fibroblast growth factor (bFGF) is a heparin-binding growth factor, so it was usually purified by affinity chromatography on a heparin-grafted support. It was previously observed that, among several modified polystyrene that exhibit anticoagulant heparin-like properties, insoluble polystyrenes modified by chlorosulphonation substituted with serine (PSSer) develop specific interactions with bFGF in solution. These PSSer resins were used as stationary phases in high-performance affinity chromatography in order to separate the radiolabelled growth factor from a bovine brain crude extract. The growth factor is strongly bound to the solid phase, as demonstrated by the adsorption isotherms. It can be adsorbed on the resin at low ionic strength and be desorbed by raising the salt concentration in the eluent. The effects of flow-rate, of the initial buffer and of the slope of the salt gradient on the adsorption and on the desorption of bFGF were investigated in order to study the thermodynamic and the kinetic parameters of the interactions and to define the optimum conditions for a fast and efficient separation of the growth factor.

INTRODUCTION

Biospecific polymers can be obtained by a chemical modification of synthetic polymers. Hence, when chlorosulphonated polystyrenes are substituted by amino acids, in particular by serine, they develop specific interactions with blood proteins[1,2]. We have previously demonstrated that this type of polymer is able to interact specifically with heparin-binding growth factors (HBGF)[3]. These compounds were

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also named fibroblast growth factors (FGFs), after the early description of their potent mitogenic activity for fibroblast[4]. Two classes of fibroblast growth factors have been described according to their isoelectric point. These closely related polypeptides, acidic and basic FGFs, have about 50% absolute homology and differ in their affinity for heparin–Sepharose[5]. The biological properties of FGFs as angiogenic[6] and wound-healing factors[7] have led to the development of fast and efficient purification methods.

We have previously shown that polystyrene substituted with serine (PSSer) exhibit a strong affinity for the basic form (bFGF). This functionalized polymer was used as a stationary phase in low-pressure affinity chromatography in order to separate and purify this growth factor[3]. Because of its mechanical properties, this support can be used in high-performance affinity chromatography (HPAC)[8,9].

Similar functional supports have also been used in the high-performance liquid affinity chromatography of serine proteases. The specific interactions presented by the active supports can be used in order to separate or purify enzymes. Moreover, HPAC was a good method for studing the interactions between the polymer and the protein in solution and to reveal the importance of kinetic parameters with respect to chromatography[10].

In this work, we determined the affinity constants of purified bFGF for PSSer using adsorption isotherms. The radiolabelled growth factor can also be eluted on the functional support, in HPAC experiments, in order to study the influence of elution parameters on the chromatographic performance of supports.

EXPERIMENTAL

Growth factors bovine brain crude extract and bFGF radiolabelled with iodine-125, were purified and prepared as described previously[3]. Polystyrene substituted with serine was prepared in two successive steps. First, insoluble copolymers styrene–divinylbenzene (2%) 200-400 mesh beads (Bio-Rad Labs., Richmond, CA, U.S.A.) are treated by chlorosulphonic acid[1]. Second, the chlorosulphonated beads are substitued with serine, as described previously[3]. Sulphonate and amino acid sulphamide substitution is determined by potentiometric titration and by elemental analysis. These groups are statistically distributed along the polymer chain. The general structure of the functional polymer is shown in Fig. 1.

Adsorption isotherms

Various amounts of bovine brain crude extract containing 1.2 μg/ml of bFGF were mixed with approximately 1 ng of radiolabelled bFGF with a specific activity of

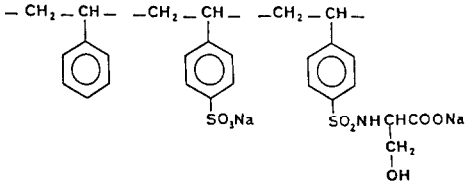

Fig. 1. Structure of functional polymer PSSer. 66% units substituted with sulphonate groups and 14% units by serine sulphamide functions.

70 000 cpm in phosphate-buffered saline (PBS)–0.4 *M* NaCl. The mixture was incubated for 1 h with 4.85 mg of PSSer resin at room temperature in a final volume of 2 ml. After incubation, the supernatant was collected and the residual radioactivity was determined in order to measure the proportion of unbounded growth factor as a function of the initial concentration of bFGF. The affinity constant was obtained using a linearization method as shown in Fig. 2[11].

High-performance affinity chromatography

The modified polystyrene beads were suspended in a 0.15 *M* NaCl–0.01 *M* PBS (pH 7.4) buffer solution and allowed to decant for 3 h. The supernatant containing fine particles was eliminated. This procedure was repeated until the supernatant was free of suspended particles, then 2 g of PSSer resin were packed in a high-performance liquid chromatographic column (5 × 0.7 cm I.D.) using a slurry method. The chromatographic system was described previously[9]. Bovine brain crude extract (1 ml) with radiolabelled bFGF was injected at room temperature using different elution rates. The adsorbed growth factor was desorbed by changing the ionic strength of the eluent. The total protein content and the amount of radiolabelled bFGF of the eluted fractions were determined. After the elution the support was washed with a 2.5 *M* NaCl–0.01 *M* PBS solution and re-equilibrated in the initial buffer.

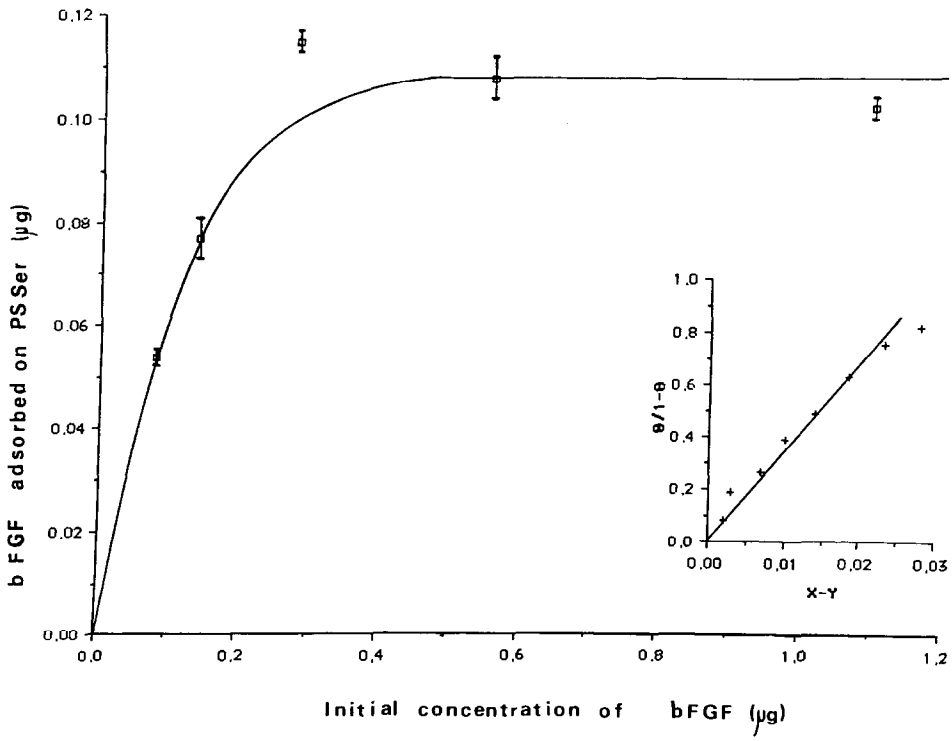

Fig. 2. Adsorption isotherm of bFGF on 4.85 mg of PSSer in 0.01 *M* phosphate buffer–0.4 *M* NaCl (pH 7.4).

RESULTS AND DISCUSSION

The composition of the resin, expressed as the percentage of monomeric units bearing the different substituted groups, is presented in Fig. 1. It was found that the polymer can develop different types of interactions with the proteins in solution[10]. It is obvious that the affinity constant relating to the adsorption isotherms is the sum of all these interactions. In fact, the hydrophobic character of the starting polymer is strongly reduced by the chlorosulphonation. However, the substantial percentage of sulphonate groups in the polymer structure confers a cation-exchange capacity to the resin. The adsorption capacity of the resin for the bFGF determined from the saturation level of the isotherm (Fig. 2) is 0.02 μg per mg of dry support. This adsorption corresponds to 2.5 ng/cm^2; the specific surface area determined by adsorption of radiolabelled serum albumin is 8.1 cm^2/mg[11]. The affinity constant of the growth factor for the functional polymer, determined from the adsorption isotherm, is 1.2 $\times$ 10^9 l/mol[11]. This thermodynamic function indicates the strong affinity of this substituted polystyrene for bFGF. Moreover, this affinity constant was determined under experimental conditions where bFGF competes with other proteins in solution.

The polystyrene resins functionalized by substitution with sulphonate and serine sulphamide residues (PSSer) are able to adsorb basic fibroblast growth factor at low ionic strength (0.4 *M* NaCl) under the HPAC elution conditions. Typical HPAC of bFGF on PSSer is shown in Fig. 3. The adsorbed growth factor can be desorbed using a linear salt gradient from 0.4 to 2 *M* with a maximum at 1.4 *M* NaCl. We can assume that the salt concentration required for the desorption of the bound growth factor reflects the strength of the interactions between bFGF and the solid surface. When the concentration of NaCl in the initial buffer is 0.5 *M*, the recovery of the bFGF in the separation (Table I) is similar to those obtained previously in the low-pressure chromatographic experiments with the same resin with a elution flow-rate of 0.5 ml/min[3]. Under these elution conditions, the pressure is *ca.* 80 bar.

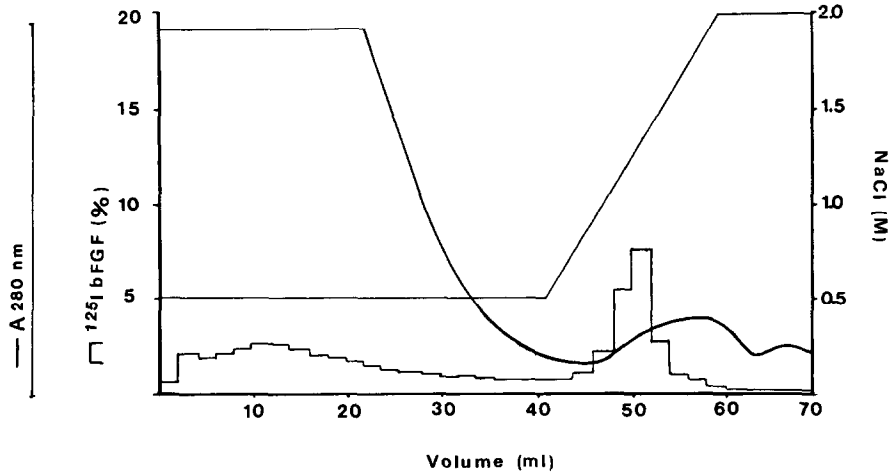

Fig. 3. Elution of radiolabelled bFGF mixed with 1 ml of bovine brain crude extract on 2 ml of PSSer support. Column, 5 $\times$ 0.7 cm I.D.; flow-rate, 0.5 ml/min; initial buffer, 0.01 *M* PBS–0.5 *M* NaCl (pH 7.4).

TABLE I

PERCENTAGE OF RADIOLABELLED bFGF RETAINED AND NON-RETAINED ON PSSer SUPPORT WITH DIFFERENT DESORPTION CONDITIONS.

Flow-rate (cm/min)	*Initial ionic strength (M NaCl)*[a]	*bFGF eluted at 0.4 M (%)*	*bFGF eluted by gradient (%)*	*Ionic strength of eluted peak (M)*
0.50	0.5	25	30	1.4
1.25	0.5	30	19	1.4
2.50	0.5	40	08	1.4
1.25	0.4	02	44	1.4

[a] Ionic strength of eluting buffer (0.01 *M*, PBS pH 7.4) in NaCl molarity.

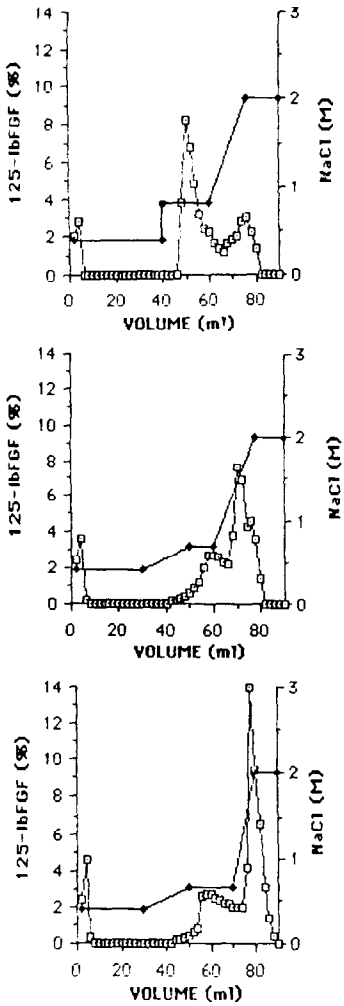

Fig. 4. Elution of radiolabelled bFGF mixed with 1 ml of bovine brain crude extract on 2 ml of PSSer support with different desorption conditions. Column, 5 × 0.7 cm I.D.; flow-rate, 0.5 ml/min; initial buffer, 0.05 *M* PBS–0.5 *M* NaCl (pH 7.4).

TABLE II

EFFECT OF INJECTED HEPARIN ON THE ADSORPTION OF RADIOLABELLED bFGF ON PSSer RESINS

Flow-rate, 0.5 ml/min (1.25 cm/min); initial buffer, PBS (pH 7.4)–0.4 *M* NaCl.

Amount of heparin injected (mg)	*bFGF eluted at 0.4 M NaCl (%)*	*bFGF desorbed at 2 M NaCl (%)*
0	6	56
0.05	8	50
4.4	71	25

The importance of the effect of elution rate on the chromatographic performance of the support is illustrated in Table I. With a low elution rate (0.2 ml/min), the amount of growth factor retained on the resin increases. This a clear indication that the heterogeneous reaction of complex formation is kinetically limited.

The ionic strength of the initial buffer is also an important parameter (Table I). When the salt concentration decreases, the percentage of bFGF retained on the solid phase increases considerably. This effect relects the importance of ionic interactions in the mechanism of adsorption. However, the complex formation can also be mediated by other components present in the injected sample.

The importance of desorption conditions on the recovery of the growth factor is demonstrated in Fig. 4. When bFGF is adsorbed on the stationary phase, it requires a considerable change in the ionic strength of the eluent for its desorption. If this change is limited, an substantial amount of growth factor remains absorbed. The salt concentration has to reach 2 *M* NaCl for the desorption and even under these conditions a percentage of growth factor remains adsorbed on the solid phase and can only be desorbed by a competitive elution.

Finally, if the sample is incubated with a small amount of heparin (50 μg), bFGF is normally adsorbed and desorbed from the active polymer (Table II). This seems to indicate that the binding site of the growth factor is not involved in the interaction with the functional polymer. However, a competive effect is observed when a large amount of heparin is injected. In fact, under these conditions, the adsorbed bFGF is completely washed out from the stationary phase. This effect probably reflects a dramatic change in the ionic interactions due to the anionic character of heparin and demonstrates the role of electrostatic interactions in the affinity of bFGF for the functional polymer.

CONCLUSION

Polystyrene resins substituted with sulphonate and serine sulphamide groups exhibit a specific and strong affinity for basic FGF in solution. The growth factor can be adsorbed on the solid phase at low ionic strength and desorbed by increasing the salt concentration as observed by HPAC. The influence of the elution rate demonstrates that the complex formation reaction between bFGF and the resin is kinetically limited. When the complex is formed, desorption can occur on increasing the ionic strength of

the eluent but a fraction of the growth factor remains adsorbed on the solid phase and can only be desorbed by a competitive elution. This adsorption is the result of different types of interactions and the importance of ionic interactions is revealed by the effect of a large amount of heparin on the chromatographic separation.

This functional polymer leads to a rapid and efficient separation of basic fibroblast growth factor and, because of the mechanical properties of the support, this separation process can easily be scaled up.

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