Journal of Chromatography, 526 (1990) 35–45
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5092

Affinity chromatography of fibroblast growth factors on substituted polystyrene

M.A. JACQUOT DOURGES* and D. GULINO

Laboratoire de Recherches sur les Macromolécules, URA 0502 CNRS, Université Paris-Nord, Avenue J.B. Clément, 93430 Villetaneuse (France)

J. COURTY, J. BADET and D. BARRITAULT

Laboratoire de Biotechnology des Cellules Eucaryotes, Université Paris, Val de Marne, Avenue Général de Gaulle, 94010 Créteil (France)

and

J. JOZEFONVICZ

Laboratoire de Recherches sur les Macromolécules, URA 0502 CNRS, Université Paris-Nord, Avenue J B Clément, 93430 Villetaneuse (France)

(First received July 13th, 1989; revised manuscript received October 26th, 1989)

SUMMARY

The heparin-binding growth factors aFGF and bFGF (acidic and basic fibroblast growth factor) from crude bovine brain extract were co-eluted with purified [^{125}I]aFGF and/or [^{125}I]bFGF as tracers from heparin-Sepharose and from several insoluble substituted polystyrenes used as stationary phases in low-pressure affinity chromatography. The ability of the resins to isolate FGFs was determined by measuring the eluted radioactivity. It was demonstrated that the various substituted polystyrene resins retain [^{125}I]aFGF and [^{125}I]bFGF with different specificities according to the chemical nature of the substituted groups bound to the polystyrene support. Bifunctional resins substituted with sulphonate and phenylalanine sulphamide groups adsorbed both [^{125}I]aFGF and [^{125}I]bFGF whereas bifunctional resins substituted with sulphonate and sulphamide serine adsorbed only [^{125}I]bFGF. These stationary phases could be adapted to high-performance affinity chromatography and used to isolate growth factors of the FGF family.

INTRODUCTION

Affinity chromatography on heparin-Sepharose has made possible the purification to homogeneity from tissues and cells of a family of growth factors

0378-4347/90/$03.50

known as heparin-binding growth factors. These compounds were also named after the early description of their potent mitogenic activity for fibroblasts [1], fibroblast growth factors (FGF) [2,3]. They have been shown to be involved in the proliferation and differentiation of a wide variety of cells derived from mesoderm and neuroectoderm such as fibroblasts and endothelial cells [3]. Two classes of fibroblast growth factors have been described according to their isoelectric points. These closely related polypeptides, acidic and basic FGFs (aFGF and bFGF) have about 50% absolute homology [4,5] and differ in their affinity to heparin-Sepharose. The biological properties of FGFs as angiogenic [6] and wound-healing factors [7,8] have led to a need to develop new techniques for their rapid and efficient purification such as affinity high-performance liquid chromatography (HPLC). We have previously shown that the binding of sulphonates and amino acid sulphamides to cross-linked polystyrene endowed this resin with significant anticoagulant heparin-like properties [9,10]. It therefore seemed useful to study whether the heparin-like properties found for these materials could be extended as regards interactions with heparin-binding growth factors. Polystyrene beads possess suitable mechanical properties and, therefore, could be used as stationary phases in the affinity HPLC of FGFs. This paper describes the purification of FGFs from bovine brain extracts using such modified polystyrene resins as affinity chromatography phases. Several of the resins bound FGFs and led to the selective elution of these growth factors.

EXPERIMENTAL

Source of growth factors

FGFs were purified from bovine brain as described [11], including fractionation with ammonium sulphate (20–60% saturated solution at 4°C), dialysis against acetic acid and then against phosphate-buffered saline (PBS)–8 m*M* Na_2HPO_4–1.5 m*M* KH_2PO_4–3.0 m*M* KCl–0.14 *M* NaCl (pH 7.4). The supernatant from centrifugation (20 000 *g*, 30 min, 4°C), referred to as the crude extract, was finally adjusted to 0.65 *M* NaCl; it contained both aFGF and bFGF.

Iodination of acidic and basic FGFs

FGFs were purified as described previously [11] and radiolabelled with ^{125}I as follows [12]: 1 μg of aFGF or bFGF was incubated with 1 mCi or 0.5 mCi of $Na^{125}I$ (CEA, Gif sur Yvette, France), respectively, and chloramine-T solution (80 μM) at room temperature in 0.1 *M* phosphate buffer (pH 7.4). The reaction was stopped 3 min later by adding sodium metabisulphite (100 μM) and sodium iodide successively. The final volume was adjusted to 500 μl by adding PBS supplemented with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) (1 mg/ml). Finally, in order to isolate [^{125}I]FGF, the samples were ultrafil-

tered using Centricon centrifugal microconcentrators (10 000 M_r cut-off; Amicon, Lexington, MA, U.S.A.).

In some experiments [^{125}I]FGFs were further chromatographed on heparin-Sepharose to eliminate non-heparin-binding FGF which was found to be biologically inactive and generated during the labelling procedure or by radiolysis. Eluted [^{125}I]FGFs were found to have retained their biological activity [13].

Preparation of substituted polystyrene resins

Polystyrene resins were substituted according to the two procedures described previously [9,10]. Insoluble styrene–divinylbenzene (2%) copolymer in the form of 200–400 mesh beads (Bio-Rad Labs., Richmond, CA, U.S.A.), were chlorosulphonated, then the chlorosulphonated beads were substituted with amino acids: L-threonine (Thr), L-alanine (Ala), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-hydroxyproline (HyPro), L-serine (Ser) and L-aspartic acid (Asp). Sulphonate and amino acid sulphamide groups were statistically distributed along the polymer chain. The general structure of such substituted polystyrenes is shown in Fig. 1. Total substitution of the different resins reached about 80% of the aromatic groups ($Y+Z$ in Table I), while the proportions of the two types of substituents, sulphonate and amino acid sulphamide (Y and Z, respectively), varied.

Heparin-Sepharose (Pharmacia, Uppsala, Sweden) was used as a standard to compare the properties of the different matrices.

Chromatography

The chromatographic columns were prepared as follows: 4 ml of heparin-Sepharose or about 5 ml of suspension of substituted resin (the dry weight of such resins depends on the chemical nature of the amino acid substituent) in PBS adjusted to 0.5 M NaCl (pH 7.4) were packed in a 40 mm × 11.4 mm I.D. column (IBF, Villeneuve la Garenne, France). Bovine brain crude extract (5 ml) containing radiolabelled FGFs (about 10^6 cpm) in the same buffer was loaded on the column and the resin was washed with 120 ml of buffer to eliminate the non-adsorbed proteins. Growth factors were eluted with a 0.5–2.5 M NaCl gradient (150 ml) at a flow-rate of 24 ml/h at room temperature. Gra-

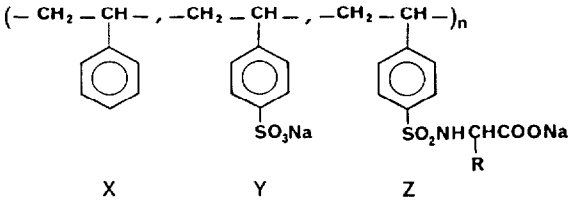

Fig. 1. General structure of the bifunctional resins substituted by a sulphonate group (Y) and an amino acid sulphamide group (Z).

TABLE I

CHEMICAL COMPOSITION OF THE DIFFERENT PSAA RESINS

PSAA = bifunctional polystyrene resin bearing sulphonate and amino acid sulphamide groups. Percentages of non-substituted units (X), units substituted with sulphonate (Y) and amino acid sulphamide (Z), respectively, were determined from the elemental analysis of nitrogen and sulphur and from acidic titration.

Resin	Substituted units[a] (%)		
	X	Y	Z
PSThr	23	61	16
PSAla	22	47	31
PSMet	22	64	14
PSPhe	23	62	15
PSPro	20	59	21
PSHyPro	20	56	24
$PSSO_3Na$	10	90	0
PSSer	20	66	14
PSAsp	23	70	7

[a] $\%X = X \cdot 100/(X+Y+Z)$; $\%Y = Y \cdot 100/(X+Y+Z)$; $\%Z = Z \cdot 100/(X+Y+Z)$.

dients were produced by a gradient former of our own design, monitored by an automatic two-valve system.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Fractions eluted from heparin-Sepharose and PSSer were analysed by SDS-PAGE (20%) according to Laemmli [14] (Phast System, Pharmacia–LKB, Uppsala, Sweden). Proteins were revealed by silver staining [15].

Bioassay

Fractions to be assayed were pooled and their salt concentration reduced to about 0.2 *M* NaCl by ultrafiltration using Centricon centrifugal microconcentrators. A 20-μl volume of each chromatographic fraction was tested for mitogenic activity on chinese hamster lung fibroblasts (CCL 39, American Type Culture Collection) by reinitiation of DNA synthesis. Subconfluent cells in 24-well plates were arrested for 24 h in serum-free medium. Fractions to be tested were injected for 24 h while 2 μCi/ml tritiated thymidine (25 Ci/mmol) were added for the last 4 h. Cells were then fixed with 10% trichloroacetic acid for 20 min at 4 °C, rinsed five times with water and lysed with 0.1 *M* NaOH (0.5 ml). The radioactivity incorporated was quantified using a scintillation counter.

RESULTS

Comparative FGF chromatographic separations using the different substituted polystyrene resins

In order to study the different resins for their ability to separate FGFs, purified [^{125}I]aFGF or [^{125}I]bFGF was added to crude bovine brain extract and used as a tracer during low-pressure chromatographic elution. [^{125}I]aFGF and [^{125}I]bFGF were eluted from a heparin-Sepharose column at the same ionic strength as their respective native forms. However, the yield of [^{125}I]FGFs

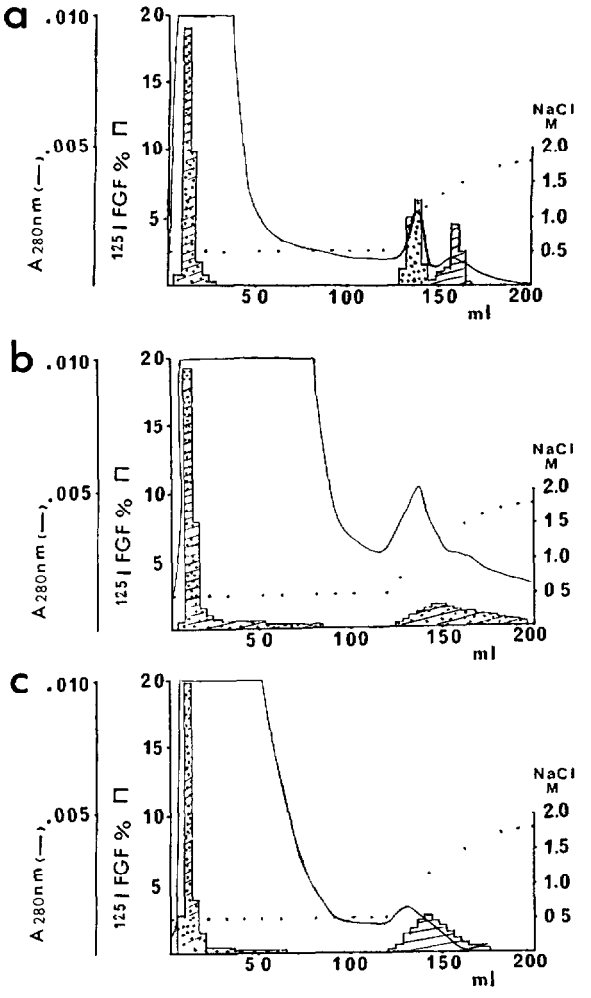

Fig. 2. Chromatography of bovine brain crude extract on (a), heparin-Sepharose, (b) PSPhe and (c) PSSer as described under Experimental. Elution profiles were monitored by absorbance determination at 280 nm and γ-counting. Dotted peaks, [^{125}I]aFGF; hatched peaks, [^{125}I]bFGF.

TABLE II

PURIFICATION OF ACIDIC FGF USING DIFFERENT RESINS

Column chromatography was monitored by measuring radioactivity NR = non-recovered, NA = non-adsorbed and RA = reversibly adsorbed [^{125}I]aFGF. M = NaCl concentration (*M*) needed to desorb FGF (top of the peak). E = efficiency of FGF purification (E = RA after chromatography on PSAA/RA after chromatography on HepS). Three different batches of radiolabelled aFGF were run on heparin-Sepharose (HepS). The efficiency of each resin was compared with the HepS involving the same batch of [^{125}I]aFGF.

Batch	Resin	NR (%)	NA (%)	RA (%)	M	E
I	$HepS_1$	38	34	28	1.0	1.00
	PSThr	40	58	2	–	0.07
	PSAla	41	56	3	1.0	0.12
	PSmet	42	50	8	1.0	0.29
	PSPhe	46	39	15	1.1	0.54
	PSPro	37	62	1	–	0.04
II	$HepS_2$	34	37	29	1.0	1.00
	PSHyPro	34	62	4	1 0	0.12
	$PSSO_3Na$	62	24	14	1.2	0.48
III	$HepS_3$	42	33	25	1.0	1.00
	PSSer	46	53	1	1.2	0.05
	PSAsp	54	42	4	1.0	0.16

retained on heparin-Sepharose was affected by the rate of radiolysis and the preparation of the labelled FGFs. Consequently, heparin-Sepharose chromatography was run as a standard in each set of experiments involving the various resins. Typical chromatograms (Fig. 2) indicate that [^{125}I]FGFs were partly retained and eluted using an NaCl gradient.

To study the substituted polystyrene matrices, the percentage of [^{125}I]FGF reversibly adsorbed on each resin was compared with that reversibly adsorbed on heparin-Sepharose, for both aFGF and bFGF. Three independent batches of [^{125}I]aFGF and [^{125}I]bFGF were chromatographed on heparin-Sepharose in order to estimate the stability of radiolabelled factors. For the three batches, the percentages of reversibly adsorbed [^{125}I]FGF were different for bFGF on heparin-Sepharose (19, 49 and 24%) whereas for aFGF they were very similar (28, 29 and 25%). aFGF was reversibly adsorbed on PSPhe and $PSSO_3Na$ (monofunctional polystyrene resin bearing sulphonate groups) columns with an efficiency of 0.54 and 0.48, respectively (Table II), whereas bFGF was better retained on the substituted polystyrene resins except for PSAla, PSHyPro, $PSSO_3Na$ and PSAsp (Table III). These results indicate a great variability in the retention capacities of the different resins for aFGF or bFGF. Heparin-Sepharose (Fig. 2a) allows the separation of both aFGF and bFGF whereas PSPhe (Fig. 2b) retains both forms without separation. PSSer seems of par-

TABLE III

PURIFICATION OF BASIC FGF USING DIFFERENT RESINS

Details as in Table II, except with bFGF instead of aFGF.

Batch	Resin	NR (%)	NA (%)	RA (%)	M	E
I	$HepS_1$	53	28	19	1.5	1.00
	PSThr	56	23	11	1.4	0.55
	PSAla	72	21	7	1.2	0.36
	PSMet	55	30	15	1.3	0.79
	PSPhe	57	30	13	1.5	0.68
	PSPro	42	48	10	0.9	0.53
II	$HepS_2$	35	16	49	1.5	1.00
	PSHyPro	27	65	8	0.7	0 16
	$PSSO_3Na$	78	8	14	1.5-2.0	0 29
III	$HepS_3$	41	35	24	1.5	1.00
	PSSer	47	30	23	1.2	0.96
	PSAsp	57	32	11	1.1	0.44

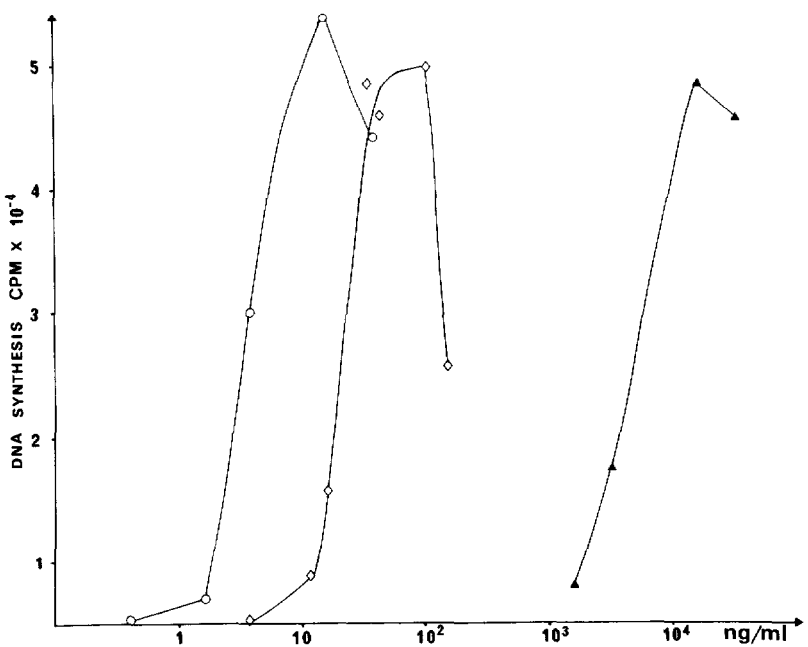

Fig. 3. Bioassay: [^{3}H]thymidine incorporation into fibroblasts in response to varying protein concentrations of (▲) crude extract and (◇) bFGF eluted from PSSer and (○) heparin-Sepharose.

ticular interest (Fig. 2c) as only bFGF was retained, with an efficiency in the same range as that of heparin–Sepharose (0.96) (Tables II and III).

Purification of bFGF on PSSer resin

PSSer resin was used for the purification of bFGF from bovine brain crude extracts under the same conditions as those described above except that larger amounts of brain extract were used (10 ml) without [^{125}I]bFGF.

Fractions eluting between 1.2 and 1.5 *M* NaCl were pooled and tested for biological activity and analysed by SDS-PAGE, whereas bFGF purified by heparin-Sepharose chromatography was the pooled fractions eluting from 1.4 to 1.6 *M* NaCl. Fig. 3 shows the dose–response curves of [^{3}H]thymidine incorporation by target CCL 39 fibroblasts stimulated by the crude extract and bFGF purified either on heparin-Sepharose or PSSer resins. The biological activity of heparin-Sepharose-purified FGF compared with the starting material indicates a purification yield of 1700 whereas PSSer resin-purified FGF was only

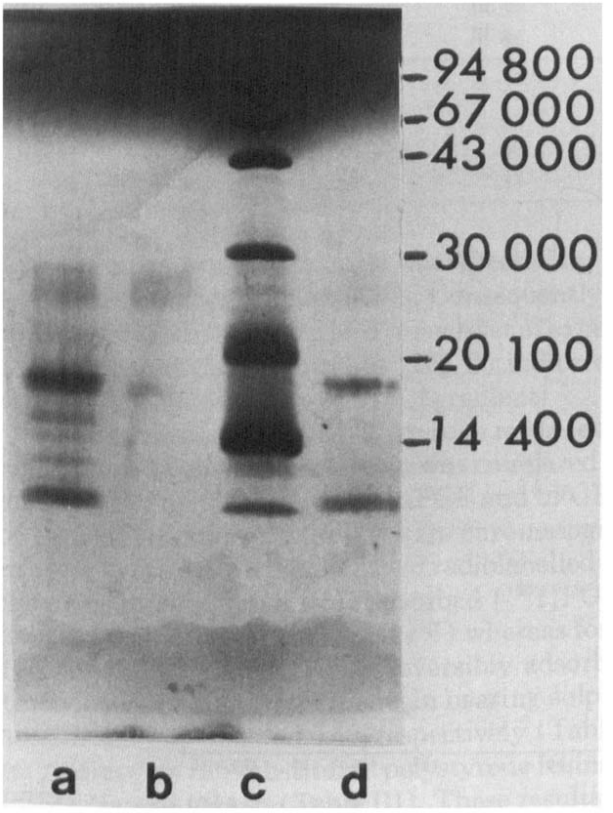

Fig. 4. SDS-PAGE of bFGF eluted from HepS (12 ng, lane b), PSSer (37 ng, lane a; 23 ng, lane d). Proteins were silver-stained. Lane c: molecular mass markers.

purified 300-fold. However, the total biological activities recovered in the two purified fractions tested were almost identical. SDS-PAGE of the purified FGF is shown in Fig. 4. PSSer chromatography yielded four contaminating bands whereas the fraction containing heparin-Sepharose-purified FGF resulted in a single band.

The protein recovery was 8 μg for heparin-Sepharose-purified FGF and 45 μg for PSSer-purified FGF.

DISCUSSION

The efficiencies of the different functional resins studied were analysed by the resolution of aFGF and bFGF elution. The yield of the purification was measured using radiolabelled growth factors and the values were compared with those obtained by heparin-Sepharose chromatography.

In a preliminary study (not shown), a few chromatographic parameters were considered. The effects of ionic strength and volume of the elution gradient on the efficiency of the resins were examined in order to optimize the affinity chromatography.

PSAA resins can be classified into three groups according to their ability to interact with FGFs (presented in Table IV). The first group, including the monofunctional polystyrene sulphonate resin and the bifunctional resins substituted with sulphonate and alanine, hydroxyproline and aspartic acid sulphamide, exhibited a poor efficiency for purifying these growth factors. The monofunctional $PSSO_3Na$ resin retained irreversibly more than 60% of FGF. As this resin has a very high content of sulphonate groups on aromatic rings (90%), the adsorption could be explained by non-specific multi-point interactions. We observed that even concentrated salt washing (2 M NaCl) did not allow adsorbed FGF to be recovered.

The resins of the second group (PSMet and PSPhe) retained both forms of

TABLE IV

ABILITY OF PSAA RESIN TO INTERACT WITH FGFS

Efficiencies are derived from Table II and III.

Group I			Group II			Group III		
Resin	Efficiency		Resin	Efficiency		Resin	Efficiency	
	aFGF	bFGF		aFGF	bFGF		aFGF	bFGF
PSAla	0.12	0 36	PSMet	0.29	0.79	PSSer	0.05	0.96
PSHyPro	0.12	0.16	PSPhe	0.54	0.68	PSThr	0.07	0.55
$PSSO_3Na$	0.48	0.29				PSPro	0.04	0.53
PSAsp	0.16	0.44						

FGF. Interestingly, aFGF and bFGF were eluted on PSPhe at 1.1 and 1.5 *M* NaCl, respectively. These conditions were identical with those observed in heparin-Sepharose chromatography. However, the elution of FGFs on PSPhe resin gave considerable overlapping, as shown in Fig. 2b, and did not result in a good separation.

Finally, the resins of the third group interacted specifically with bFGF, which was eluted with 0.9 *M* NaCl (PSPro), 1.2 *M* NaCl (PSSer) and 1.4 *M* NaCl (PSThr). However, we observed that the efficiency obtained with PSSer was almost unit. This result is of particular interest for the purification of bFGF, which is present in very low concentrations in crude bovine brain extract.

From these comparative studies we conclude that the retention capacity of the functional resins for FGFs varies with the chemical nature of the substituted amino acid on the polystyrene matrix. Interestingly, when aFGF is retained on the resin (groups I and II), it is eluted at the same ionic strength (1 *M* NaCl), whereas the ionic strength of the elution of bFGF varies according to the resins. Furthermore, changes in the amino acids (i.e., hydroxyproline versus proline) modify completely the performance of the column although the substitution yields were similar (see Table I). The degree of amino acid substitution did not seem to be important as resins bearing threonine, methionine, phenylalanine and serine with similar composition ($Z=16$, 14, 15 and 14%) showed very different chromatographic performances.

As PSSer resin was found of particular interest for the purification of bFGF, we studied the performance of this phase for purifying biologically active bFGF. bFGF purified with PSSer was biologically active although not as pure as heparin-Sepharose-purified bFGF. However, we did not optimize the elution conditions. The broadness of the elution peak (1.2–1.5 *M* NaCl) obtained in the PSSer chromatography may account for the presence of contaminants revealed in the electrophoretic pattern and by a higher protein content in this fraction compared with heparin-Sepharose-purified bFGF.

CONCLUSION

The specificity of the interaction of aFGF or bFGF with polystyrene substituted with sulphonate groups and various amino acid sulphamide groups remains to be understood. The implication of serine, proline and threonine correlated with the strong interaction between the corresponding resin and bFGF and moreover with the absence of affinity of the resin for aFGF is clear. This suggests a possible implication of peptide sequences containing these amino acids in specific recognition sites of bFGF.

It has been shown that PSSer resin could be used as an efficient resin for the purification of bFGF. Several assays of crude bovine brain extracts allowed

the purification of bFGF in a very reproducible yield over a long period of time. This resin could be easily adapted for HPLC. However, more work needs to be done for optimization of the method.

ACKNOWLEDGEMENTS

This work was supported by the Association Nationale pour la Valorisation de la Recherche (ANVAR) and Institut Choay (Paris, France).

REFERENCES

1 H.A. Armelin, Proc. Natl. Acad. Sci. U.S.A., 70 (1973) 2702.
2 K.A. Thomas and G. Guimenez-Gallego, Trends Biochem. Sci., 11 (1986) 81.
3 D. Gospodarowicz, G. Neufeld and L. Schweigerer, Mol. Cell. Endocrinol., 46 (1986) 187.
4 D.J. Strydom, J.W. Harper and R.R. Lobb, Biochemistry, 25 (1986) 945.
5 F. Esch, N. Ueno, A. Baird, F. Hill, L. Denoroy, N. Ling, D. Gospodarowicz and R. Guillemin, Biochem. Biophys. Res. Commun., 133 (1985) 554.
6 J. Folkman and M. Klagsburn, Science, 235 (1987) 442.
7 J.M. Davidson, M. Klagsburn, X.E. Hall, A. Buckley, R. Sullivan, P.S. Brener and S.C. Woodward, J Cell Biol., 100 (1985) 1219.
8 G. Petrousos, J. Courty, R. Guimares, Y. Pouliquen, D. Barritault, J. Plouet and Y. Courtois, Curr. Eye Res., 3 (1987) 593.
9 C. Fougnot, J Jozefonvicz, M. Samama and L. Bara, Ann. Biomed. Eng., 7 (1979) 429.
10 C. Fougnot, M.P. Dupillier and M. Jozefowicz, Biomaterials, 4 (1983) 101.
11 J. Courty, C. Loret, M. Moenner, B. Chevallier, O. Lagente, Y. Courtois and D. Barritault, Biochimie, 67 (1985) 265.
12 W.M. Hunter and F.C. Greenwood, Nature (London), 194 (1962) 495.
13 M. Moenner, B. Chevallier, J. Badet and D. Barritault, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 5024.
14 U.K. Laemmli, Nature (London), 277 (1970) 680.
15 J. Henkeshoven and R. Dernick, Electrophoresis, 6 (1985) 103.