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USE OF SIZE-EXCLUSION AND ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ISOLATION OF BIOLOGICALLY ACTIVE GROWTH FACTORS

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SUMMARY

Size-exclusion and ion-exchange high-performance liquid chromatography were used to purify biologically active growth factors as measured by the ability of the factors to stimulate DNA synthesis in 3T3 cells. Chromatography was performed in aqueous buffer and at neutral pH to avoid possible inactivation of biological activity. The growth factors analyzed were chondrosarcoma growth factor (CHSA-GF), human milk growth factor (HMGF), retinal-derived growth factor (RDGF) and mouse epidermal growth factor (EGF). CHSA-GF, HMGF, and RDGF were eluted from TSK 2000 columns as well-defined peaks of activity with molecular weights of 12,000–15,000, 5000–6000, and 16,000–18,000, respectively. EGF was found to have an abnormally low molecular weight after chromatography on TSK 2000. However, incorporation of guanidine · HCl into the TSK column resulted in the elution of EGF at its known molecular weight of *ca.* 6000. Anion-exchange high-performance liquid chromatography on AX 300 was used for the purification of HMGF and RDGF, and cation-exchange high-performance liquid chromatography on CM 300 was used for the purification of CHSA-GF. The results show that size-exclusion and ion-exchange chromatography can be used without organic solvents or extremes in pH to purify a number of different growth factors successfully with retention of biological activity.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a promising method for the characterization and purification of polypeptide growth factors. HPLC on reversed-phase columns has been used to isolate epidermal growth factor (EGF), sarcoma growth factor (SGF) and transforming growth factor (TGF)[1–3]. In our work on the purification of growth factors from chondrosarcoma (CHSA), we have found that there are limitations to the use of reversed-phase liquid chromatography. For example, organic modifiers used conventionally in reversed-phase liquid chromatography, such as acetonitrile and 1-propanol, inactivate growth-factor activity when

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used at concentrations higher than 20%. In addition, the commonly used ion-pairing agent, trifluoroacetic acid, which produces a pH of 2, inactivates CHSA-derived growth-factor activity. Since organic solvents and extremes in pH often denature proteins and inactivate biological activity, it is important to develop HPLC techniques under non-denaturing conditions to isolate biologically active polypeptides, such as growth factors.

Recently, size-exclusion and ion-exchange columns have become available for HPLC applications. For example, cation-exchange HPLC with pyridine has been used to purify TGF[4]. In this report, we demonstrate that chondrosarcoma-derived growth factor (CHSA-GF)[5] can be purified by HPLC size-exclusion and ion-exchange columns, equilibrated and eluted with aqueous buffers. These HPLC techniques are generally applicable and can be used to isolate other biologically active growth factors such as EGF[6] and growth factors found in the retina (RDGF)[7] and in human milk (HMGF)[8,9].

EXPERIMENTAL

Growth factors

Mouse epidermal growth factor (EGF) was purchased from Collaborative Research (Waltham, MA, U.S.A.). HMGF was isolated from human milk, as previously described[8,9]. Briefly, human milk is centrifuged to remove fats, cells, and debris. The skimmed milk fraction is acidified to pH 4.3 and the precipitate that forms is removed by centrifugation. The supernatant is fractionated by gel chromatography on Sephadex G-100. Three peaks of growth-factor activity are obtained. The major peak contains *ca.* 75% of the total growth-factor activity and has a molecular weight of *ca.* 6000. This peak is designated as HMGF. Retinal-derived growth factor (RDGF) was isolated from bovine retina, as previously described[7]. Briefly, retinas are dissected from bovine eyes and are extracted in balanced salt solution for 3 h. The pH of the crude extract is adjusted to 4, and the precipitate formed is removed by centrifugation. The crude extract is further extracted repeatedly with 90% ethanol. The ethanol-insoluble material is resuspended in water and is designated as RDGF. Chondrosarcoma-derived growth factor (CHSA-GF) was isolated from chromatin, as previously described[5]. Briefly, a transplantable rat chondrosarcoma is fractionated into cellular and extracellular matrix fractions by collagenase digestion. Chromatin is prepared from the cells, and chromosomal proteins are extracted by incubation with sodium chloride. The proteins that are extracted between 0.25 *M* and 0.75 *M* are further fractionated by cation-exchange chromatography on Bio-Rex 70. The active peak of growth factor activity is designated as CHSA-GF.

Chemicals

Sequanal-grade guanidine hydrochloride was purchased from either Schwarz/Mann (Spring Valley, NY, U.S.A.) or Pierce (Rockford, IL, U.S.A.). HPLC-grade water was prepared by a Hydro Organic Adsorption-Deionization System (Hydro Ultrapure Water Systems, Weymouth, MA, U.S.A.).

HPLC system

Chromatography was performed with a Beckman Model 332 gradient liquid

chromatography system, which consists of two Model 110A pumps controlled by a Model 420 system controller-programmer. Samples were applied to the columns with an Altex Model 210 injection valve, and the eluted proteins were monitored with an Altex Model 155-40 variable-wavelength spectrophotometer at either 220 or 280 nm. All experiments were performed at room temperature.

Size-exclusion HPLC

Size-exclusion HPLC of HMGF, RDGF, and CHSA-GF was performed under nondenaturing and denaturing conditions. As much as 2 mg of lyophilized growth-factor preparations were reconstituted in 100 μl of the appropriate buffer and centrifuged at 12,000 *g* to remove insoluble material. The clarified supernatants were applied to the columns by injection with a 100-μl sample loop and eluted at flow-rates of either 0.5 or 1.0 ml/min.

Chromatography under non denaturing conditions was performed on a TSK 2000 column (60 cm × 7.5 mm I.D., Altex) equilibrated with 0.05 *M* potassium phosphate, 0.1 *M* ammonium sulfate, pH 7.0. Fractions (0.8 ml) were collected and tested directly for their ability to stimulate DNA synthesis in 3T3 cells. The following proteins were used as markers to calibrate the column: blue dextran (mol.wt. $2 \cdot 10^6$), β-galactosidase (mol.wt. 130,000), phosphorylase b (mol.wt. 94,000), ovalbumin (mol.wt. 43,000), α-chymotrypsinogen (mol.wt. 25,700), myoglobin (mol.wt. 17,800), and insulin (mol.wt. 5800).

Chromatography under denaturing conditions was performed on a TSK 3000 column (50 cm × 8 mm I.D., Varian), equilibrated with 6 *M* guanidine hydrochloride, 0.02 *M* 2-(N-morpholino)ethanesulfonic acid, 5 m*M* dithiothreitol, pH 6.5. Fractions were collected and dialyzed exhaustively against deionized distilled water before being tested for their ability to stimulate DNA synthesis. The following proteins were used as markers to calibrate the column: blue dextran (mol.wt. $2 \cdot 10^6$), ovalbumin (mol.wt. 43,000), myoglobin (mol.wt. 17,800) and insulin (mol.wt. 5800, subunit molecular weight *ca.* 3000).

Cation-exchange HPLC

Chromatography of CHSA-GF was performed on a Synchropak CM 300 column (250 × 4.1 mm I.D., SynChrom, Linden, IN, U.S.A.) The column was prewashed with 30 ml of 1.5 *M* sodium chloride, 0.01 *M* Tris · HCl, pH 7.0, for 1 h and then equilibrated with 30 ml of 0.1 *M* sodium chloride, 0.01 *M* Tris · HCl, pH 7.0. As much as 5 mg of lyophilized CHSA-GF was reconstituted in 2 ml of the equilibration buffer or a preparation of CHSA-GF was dialyzed against the equilibration buffer and concentrated to a convenient concentration (2.5 mg/ml) with an Amicon Model 52 stirred cell. The samples were centrifuged at 12,000 *g* in order to remove any insoluble material, and applied to the column with a 2-ml sample loop. Sample volumes greater than 2 ml required multiple injections. After all the unretained protein was eluted, as determined by monitoring at 220 or 280 nm, a 30-ml gradient from 0.1 to 1.5 *M* sodium chloride buffered with 0.01 *M* Tris · HCl (pH 7.0), was applied over a period of 60 min. The flow-rate was 0.5 ml/min. Fractions of 0.5 ml were collected and assayed directly on 3T3 cells for their ability to stimulate DNA synthesis.

Anion-exchange HPLC

Chromatography of HMGF and RDGF was performed on a Synchropak AX 300 column (250 × 4.1 mm I.D., SynChrom). The column was prewashed with 30 ml of 1.5 *M* sodium chloride, 0.01 *M* sodium acetate, pH 5.6, for 1 h, followed by equilibration with 30 ml of 0.01 *M* sodium acetate, pH 5.6. Growth factor was prepared for application to the column in the same manner as described above for the cation-exchange column. After all the unretained protein was eluted, a 30-ml gradient from 0 to 1.0 *M* sodium chloride, buffered with 0.01 *M* sodium acetate (pH 5.6), was applied over a period of 60 min. Fractions of 0.5 ml were collected at a flow-rate of 0.5 ml/min and were assayed directly for the ability to stimulate DNA synthesis in 3T3 cells.

DNA synthesis in 3T3 cells

Methods for growing the BALB/C 3T3 cells, for preparing confluent, quiescent 3T3 cells in 96-well microtiter plates, and for assaying DNA synthesis by measuring the incorporation of [methyl-^{3}H]thymidine into DNA have been described previously[10]. Briefly, a microtiter plate, containing *ca.* $2 \cdot 10^4$ confluent, quiescent BALB/C 3T3 cells per microtiter well was prepared. The sample and [^{3}H]thymidine (4 μCi/ml) were added, and the incorporation of [^{3}H]thymidine into DNA was measured after a 30–40-h incubation period. In the bioassay, background incorporation is typically 200–1000 cpm and maximal incorporation is 120,000–150,000 cpm.

RESULTS

Size-exclusion HPLC

Chromatography of CHSA-GF, RDGF, and HMGF was performed on a HPLC TSK 2000 size-exclusion column. As shown in Fig. 1, CHSA-GF (a), RDGF (b) and HMGF (c) were eluted as well-defined peaks of growth-factor activity, as measured by the stimulation of incorporation of [^{3}H]thymidine into the DNA of 3T3 cells. The estimated molecular weights were 12,000–15,000 (CHSA-GF), 5000–6000 (HMGF), and 16,000–18,000 (RDGF). Biological activity was recovered in yields of 30% (CHSA-GF), 35% (HMGF) and 75% (RDGF), (Table I).

EGF, having a known molecular weight of *ca.* 6000, was found to be eluted well after the insulin (mol.wt. 5800) calibration marker (Fig. 2a). As this result suggested that EGF was interacting with the TSK 2000 column in some manner resulting in retarded elution, EGF was analyzed on a TSK 3000 column equilibrated with 6 *M* guanidine · HCl, 5 m*M* dithiothreitol, pH 6.5. Under these conditions, EGF was eluted from the TSK column as a well-defined peak, corresponding to its correct molecular weight of 6000 (Fig. 2b). Incorporation of guanidine · HCl into the TSK column did not have any adverse effects on the biological activity of EGF. Guanidine · HCl was also compatible with the growth factor activities of CHSA-GF, RDGF, and HMGF (Fig. 3). In the presence of guanidine · HCl and dithiothreitol, CHSA-GF (Fig. 3a), RDGF (Fig. 3b) and HMGF (Fig. 3c) were found to remain biologically active and to be eluted at the same molecular weights and with approximately the same recoveries (Table I) as in their absence (Fig. 1).

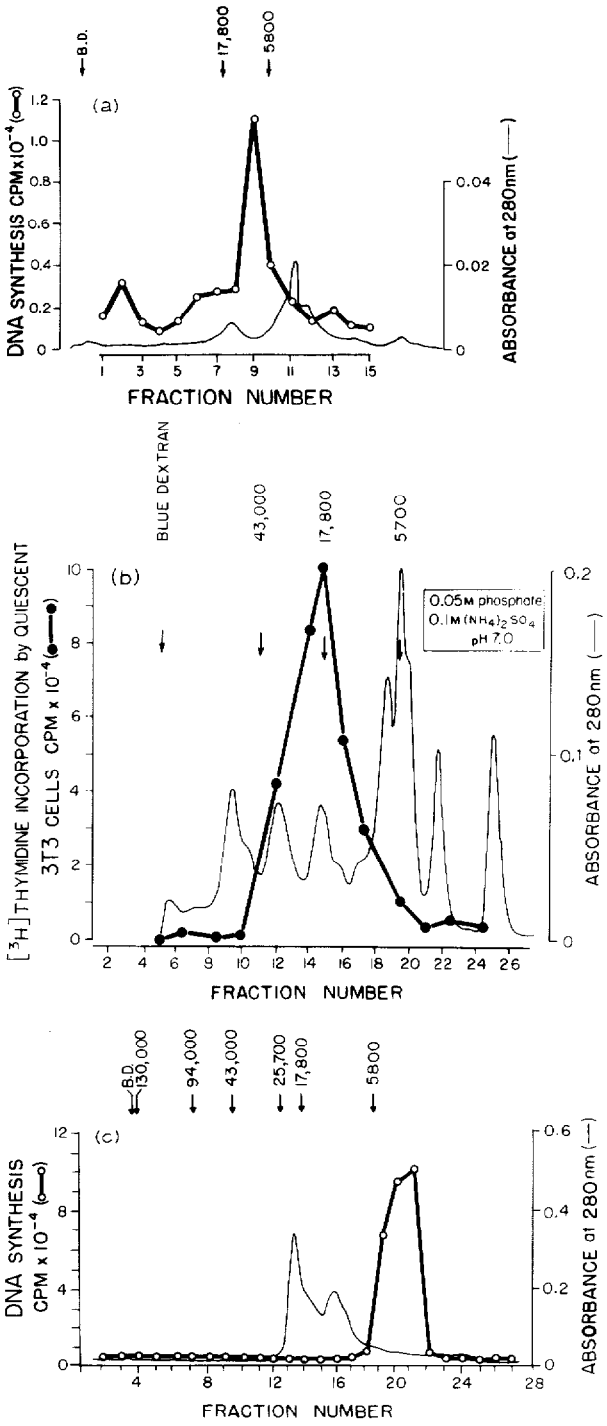

Fig. 1. Size-exclusion HPLC of CHSA-GF (a), RDGF (b), HMGF (c) was performed on a TSK 2000 column (60 cm × 7.5 mm I.D., Altex) equilibrated with 0.05 *M* potassium phosphate, 0.1 *M* ammonium sulfate, pH 7.0. Fractions of 0.8 ml were collected at a flow-rate of 0.5 ml/min. Aliquots were assayed directly for their ability to stimulate DNA synthesis in 3T3 cells. B.D. = Blue dextran.

TABLE I

RECOVERY OF BIOLOGICAL ACTIVITY

	Recovery (%)		
	CHSA-GF	*RDGF*	*HMGF*
Size-exclusion HPLC			
A. Phosphate buffer, pH 7.0	30	75	35
B. Guanidine · HCl, pH 6.5	30	72	15
Ion-exchange HPLC			
A. Cation exchange	20	—	—
B. Anion exchange	—	60	30

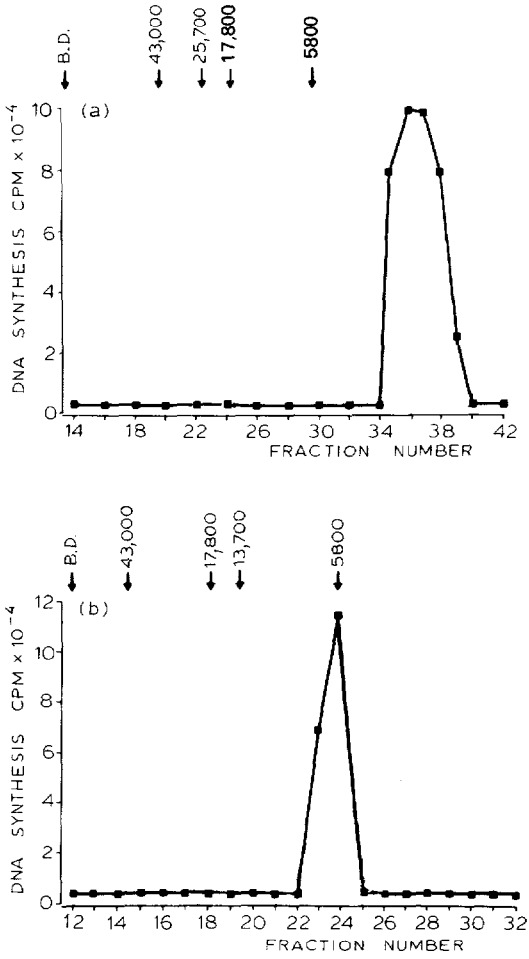

Fig. 2. Size-exclusion HPLC of EGF. (a) EGF was analyzed on a TSK 2000 column equilibrated with 0.05 *M* potassium phosphate, 0.1 *M* ammonium sulfate, pH 7.0. (b) EGF was analyzed on a TSK 3000 column equilibrated with 6 *M* guanidine · HCl, 0.02 *M* 2-(N-morpholino)ethanesulfonic acid (MES), 5 m*M* dithiothreitol, pH 6.5. Fractions of 0.8 ml were collected at a flow-rate of 0.5 ml/min at room temperature. Fractions were either tested directly on 3T3 cells (Fig. 2a) or dialyzed exhaustively against distilled water (Fig. 2b) prior to testing for the ability to stimulate DNA synthesis in 3T3 cells.

Ion-exchange HPLC

Fig. 4 shows the elution profile of CHSA-GF, chromatographed on a CM 300 cation-exchange column. Over 90% of the total protein applied on the column was unretained by the resin. However, all of the CHSA-GF biological activity was retained and could be eluted from the column as a well-defined peak with a linear sodium chloride gradient from 0.1 to 1.5 *M*. Biological activity was eluted at *ca.* 0.8 *M* sodium chloride and 20% of the original biological activity is recovered (Table I).

Fig. 5 shows the HPLC elution profiles of HMGF (a) and RDGF (b), respectively, chromatographed on an AX 300 anion-exchange column. Application of

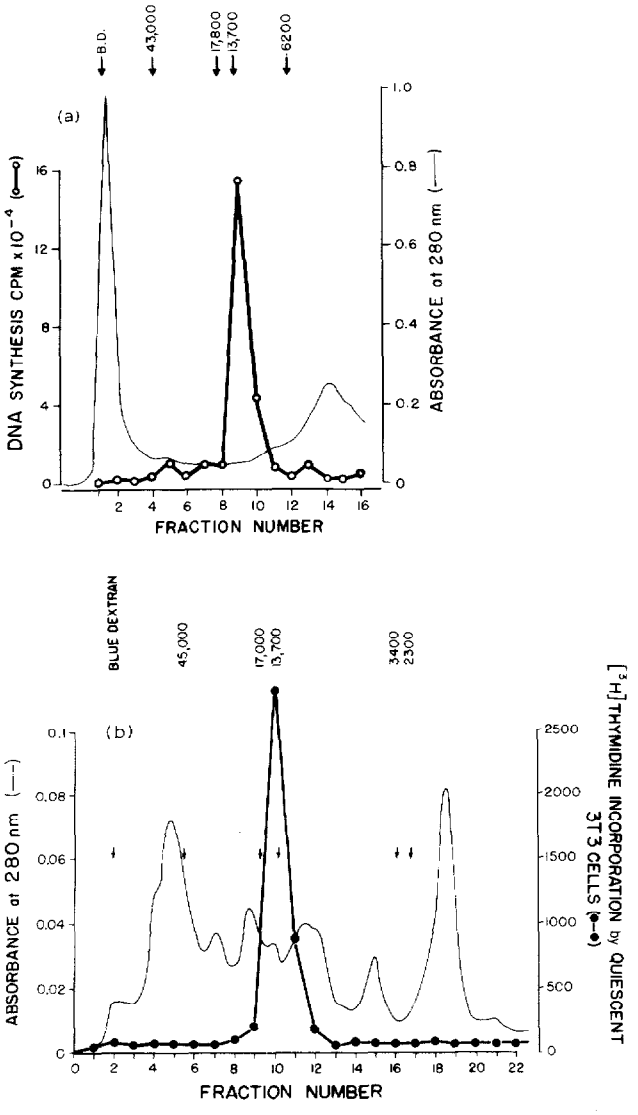

Fig. 3.

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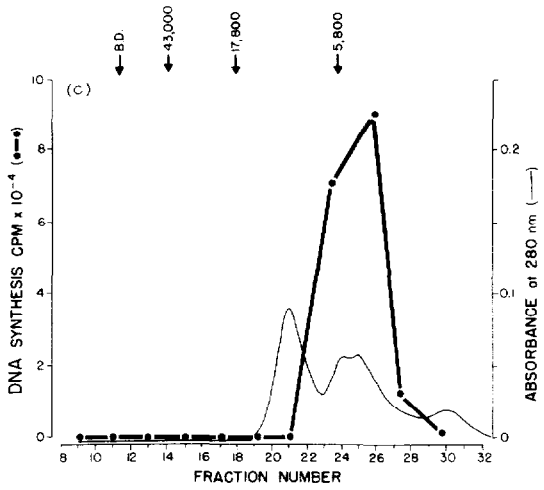

Fig. 3. Size-exclusion HPLC in guanidine · HCl. Size-exclusion HPLC of CHSA-GF (a), RDGF (b) and HMGF (c) was performed on a TSK 2000 column 50 cm × 8 mm I.D., Varian) equilibrated with 6 *M* guanidine, 0.02 *M* MES, 5 m*M* dithiothreitol, pH 6.5. Fractions of 0.8 ml were collected at a flow-rate of 0.5 ml/min at room temperature. The fractions were dialyzed exhaustively against deionized distilled water, and assayed for the ability to stimulate DNA synthesis in 3T3 cells.

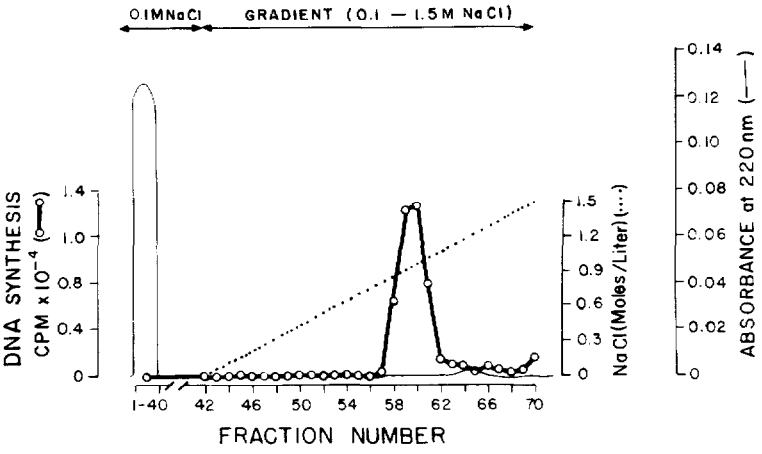

Fig. 4. Cation-exchange HPLC of CHSA-GF purified as described in Fig. 3a was performed at room temperature on a Synchropak CM 300 column (250 × 4.1 mm I.D., SynChrom) equilibrated with 0.1 *M* sodium chloride, 0.02 *M* Tris · HCl, pH 7.0. Growth-factor activity retained by the resin was eluted from the column with a 30-ml gradient from 0.1 to 1.5 *M* sodium chloride in 0.01 *M* Tris · HCl, pH 7.0, over a period of 60 min. Fractions were collected at 0.5 ml/min and assayed directly for their ability to stimulate DNA synthesis in 3T3 cells.

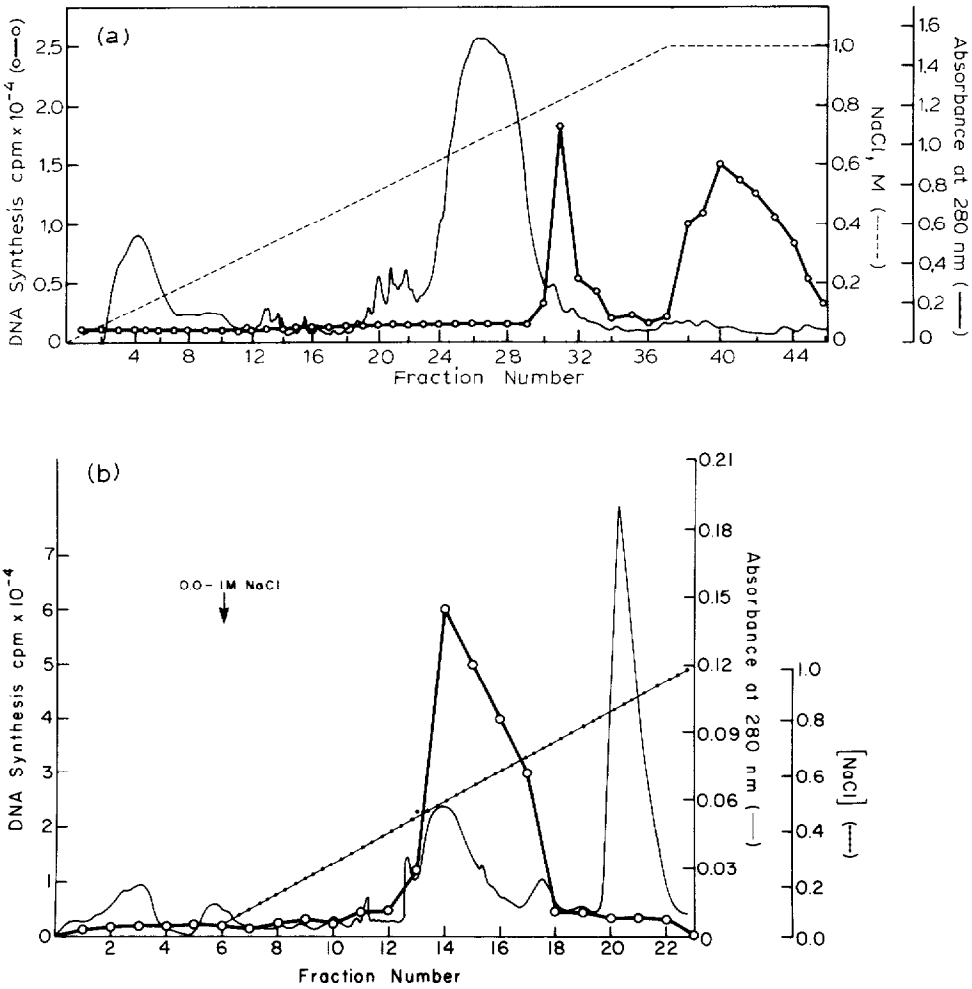

Fig. 5. Anion-exchange HPLC of HMGF (a) and RDGF purified as described in Fig. 3b (b) was performed at room temperature on a Synchropak AX 300 column (250 × 4.1 mm I.D., SynChrom) equilibrated with 0.01 *M* sodium acetate, pH 5.6. Growth-factor activity retained by the resin was eluted from the column with a 30-ml gradient from 0 to 1.0 *M* sodium chloride in 0.01 *M* sodium acetate, pH 5.6, over a period of 60 min. Fractions were collected at 0.5 ml/min and assayed directly for their stimulation of DNA synthesis in 3T3 cells.

HMGF to the AX 300 column was immediately followed by a linear sodium chloride gradient from 0 to 1 *M* (Fig. 5a). The protein profile showed two major peaks of protein, eluted at *ca.* 0.1 and 0.6 *M* sodium chloride, respectively, with several smaller peaks in between. However, the HMGF biological activity was effectively separated from these contaminating proteins as two peaks, one at *ca.* 0.8 *M* sodium chloride and the other at 1.0 *M* sodium chloride. About 30% of the original activity can be recovered (Table I). The elution profile of RDGF (Fig. 5b), chromatographed on the AX 300 column, shows that most of the protein was retained by the resin. Application of a linear sodium chloride gradient from 0 to 1 *M* resulted in good resolution of the

protein mixture, the biological activity being eluted as a single, distinct peak within a range of 0.5–0.65 *M* sodium cloride. About 60% of the original biological activity was recovered (Table I).

DISCUSSION

HPLC is a powerful tool for the purification and characterization of polypeptides. In general, many of the HPLC applications described for polypeptide purification have made use of organic solvents and extremes in pH, conditions which lead to protein denaturation, and in some cases to inactivation of biological activity. Growth factors are polypeptides the biological activity of which is the stimulation of cellular proliferation. Reversed-phase and ion-exchange HPLC in the presence of organic solvents, such as acetonitrile, 1-propanol, and pyridine have been used to purify a class of growth factors that bind to EGF receptors, including SGF, TGF and EGF[1–4]. These methods often use trifluoroacetic acid at pH 2. As an alternative to exposing growth factors to such harsh conditions of solvent and low pH, we have characterized and purified a number of structurally dissimilar growth factors by size-exclusion and ion-exchange HPLC, performed in the presence of aqueous buffers. The four growth factors analyzed, CHSA-GF (mol.wt. 12,000–15,000, p*I* 9–10), RDGF (mol.wt. 16,000–18,000, p*I* 4–5), HMGF (mol.wt. 5000–6000, p*I* 4–5) and EGF (mol.wt. 6100, p*I* 4.6), were recovered from both HPLC columns as well-defined peaks exhibiting biological activity.

Size-exclusion HPLC, performed on the TSK columns, has the advantage over conventional gel filtration methods of speed, high resolution, and high sensitivity for the detection of proteins. In addition, the ease with which a column can be repeatedly calibrated permits more accurate determinations of molecular weights. Cation- and anion-exchange HPLC provide separations of growth-factor activity from contaminating proteins that are as good as if not better than the conventional ion-exchange techniques.

Another technique for growth-factor purification is the addition of guanidine · HCl to TSK size-exclusion columns. Guanidine · HCl, buffered to pH 6.5, does not appear to affect growth factor activity adversely and offers some advantages. First, protein samples resuspended in the 100-μl sample volume used for loading HPLC TSK columns are more soluble in guanidine than in ordinary buffers. Thus losses due to insolubility can be minimized. More importantly, in determining the molecular weights of growth factors and other polypeptides, artifacts might arise from the interaction of the polypeptide and the silica gel of the TSK column. For example, EGF is retarded on TSK columns and is eluted as if substantially below its known molecular weight. However, when guanidine · HCl is incorporated into the TSK column, EGF is eluted at its correct molecular weight, namely 6000. The other three growth factors analyzed on TSK columns in the presence of guanidine · HCl, CHSA-GF, RDGF and HMGF, elute with the same molecular weights and with comparable recoveries as in its absence.

In summary, size-exclusion and ion-exchange HPLC in the presence of neutral aqueous buffers are promising methods for the purification of native, biologically active polypeptides.

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REFERENCES

1 L. M. Matrisian, B. R. Larsen, J. S. Finch and B. E. Magun, *The American Socíely for Cell Biology, 21st Annual Meeting, Nov. 9–13, 1981.*
2 M. A. Anzano, A. B. Roberts, J. M. Smith, L. C. Lamb and M. B. Spron, *Anal. Biochem.*, 125 (1982) 217–224.
3 A. B. Roberts, M. A. Anzano, L. C. Lamb, J. M. Smith, C. H. Frolik, H. Marquardt, G. J. Todaro, and M. B. Sporn, *Nature (London)*, 295 (1982) 417–419.
4 C. A. Frolik, L. L. Dart and M. B. Sporn, *Anal. Biochem.*, 125 (1982) 203–209.
5 R. C. Sullivan, J. C. Azizkhan, Y. Shing and M. Klagsbrun, *J. Cell. Physiol.*, submitted for publication.
6 S. Cohen, *J. Biol. Chem.*, 237 (1962) 1555–1562.
7 P. A. D'Amore, B. M. Glaser, S. K. Brunson and A. H. Fenselau, *Proc. Nat. Acad. Sci. U.S.*, 78 (1981) 3068–3072.
8 M. Klagsbrun, *Proc. Nat. Acad. Sci. U.S.*, (1978) 5057–5061.
9 Y. Shing and M. Klagsbrun, *Methods Molec. Cell Biol.*, (1983) in press.
10 M. Klagsbrun, R. Langer, R. Levenson, S. Smith and C. Lillehei, *Exp. Cell Res.*, 105 (1977) 99–108.