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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LXXIV*. SEPARATION OF HEPARIN-BINDING GROWTH FACTORS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of several immunologically related forms of the bovine brain basic heparin-binding growth factor by reversed-phase high-performance liquid chromatography is described. With Bakerbond C_4 reversed-phase columns, it is possible to resolve the 0.8–1.0 *M* sodium chloride and the 1.0–1.3 *M* sodium chloride components from the preceding heparin-Sepharose affinity chromatographic step in the purification procedure for these polypeptide mitogens into multiple active forms, all of which exhibit similar molecular weights and immunoreactivity with specific polyclonal antisera. Structural characterisation suggests that it is likely that these forms represent different stages in the post-translational processing of these polypeptide mitogens.

INTRODUCTION

Malignant cells are characterised by their ability to multiply in a relatively autonomous manner. To explain this, it has been suggested[2,3] that transformation results from (i) an increased production of autocrine growth factors, (ii) amplification of the mitogenic signal resulting from an increase in membrane receptors, and (iii) changes in post-receptor signal transduction pathways through the participation of, *inter alia*, transformation proteins. Similarly, normal cell differentiation and proliferation involves the interplay of two classes of growth factors: competence factors, which mediate the passage of cells through the G_0/G_1 phase, and progression factors which, foster the passage through the S phase. Earlier studies from this and other laboratories have pointed to the existence of particular growth factor activity in brain, pituitary, and other mesoderm-derived tissues. Two families of polypeptide growth factors, which exhibit specific affinity for heparin and certain biomimetic dyes, have been subsequently isolated[4–7] from bovine, murine, and porcine tissues. The present paper describes our experiences pertinent to the characterisation of the

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basic, heparin-binding growth factor (HBGF)* from bovine brain by reversed-phase high-performance liquid chromatography (RP-HPLC) and associated sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Purification of bovine brain heparin-binding growth factor

All purification procedures were performed below 4°C. Bovine brains (1.5 kg) were homogenized in 0.1 *M* ammonium sulphate, containing 1 m*M* ethylenediaminetetraacetic acid (EDTA), 2 m*M* phenylmethylsulphonylfluoride (PMSF), 2 μg/ml leupeptin and pepstatin. The crude homogenate was adjusted to pH 3.5 and immediately centrifuged at 6500 *g* for 30 min. The supernatant was adjusted to pH 7.0, 290 g/l ammonium sulphate was added, and the preparation was centrifuged at 6500 *g* for 30 min. Ammonium sulphate (250 g/l) was added to the resulting supernatant which was then centrifuged as before. The final precipitate was suspended in, and dialysed against, distilled water for 24 h. The dialysant was lyophilized to dryness, dissolved in 0.1 *M* sodium chloride, 0.1 *M* sodium dihydrogenphosphate (pH 6) (buffer A) and clarified by centrifugation for 5 min at 5000 *g*. The supernatant was then loaded onto a CM-Sephadex C-50 cation-exchange column (250 ml bed volume) at 1.5 ml/min. The column was washed with buffer A, then with 0.15 *M* sodium chloride, 0.1 *M* sodium dihydrogenphosphate (pH 6) (buffer B), and finally with 0.6 *M* sodium chloride, 0.1 *M* sodium dihydrogenphosphate (pH 6) (buffer C). Fractions eluted by buffer C that had high UV absorbances and that contained most of the mitogenic activity were pooled and loaded onto a heparin-Sepharose affinity column (30 ml bed volume) at 0.5 ml/min. After washing the column first with 0.6 *M* sodium chloride, 10 m*M* Tris–HCl, (pH 7), and then with 0.8 *M* sodium chloride, 10 m*M* Tris–HCl (pH 7), the bound proteins were eluted with a 0.8–2.0 *M* sodium chloride, 10 m*M* Tris–HCl (pH 7) gradient. Fractions collected by gradient elution were assayed for mitogenic activity with the 3T3-cell assay, and discrete zones of bioactivity were pooled as appropriate. Aliquots were kept for electrophoresis and Western immunoblots, while the remainder of the samples was used for RP-HPLC and structural studies.

High-performance liquid chromatographic techniques

A Waters Model 224 liquid chromatograph (Milford, MA, U.S.A.) equipped for gradient elution (two M6000A pumps, a M660 gradient programmer and a Model 450 variable-wavelength detector) was employed. Chromatographic separations were performed on *n*-butyl Bakerbond stationary phases (Baker, Phillipsburg, NJ, U.S.A.) packed into 15 × 0.46 cm I.D. columns. Fractions were collected with a Pharmacia

* Pituitary, brain and other tissues contain several families of structurally distinct polypeptide mitogens or growth factors. One family, with the ability to cause proliferation of mesodermally derived cells, has traditionally been assayed via incorporation of [^{3}H]thymidine into 3T3 fibroblasts and consequently has been called "fibroblast growth factors". However this nomenclature is unsatisfactory on both structural and functional grounds because of the general responsiveness of 3T3 fibroblasts to many mitogens. In view of the propensity of this family of polypeptide growth factors to bind to heparin —a binding phenomenon of relevance to the biologically important ligand, heparin sulphate/proteoglycans— the nomenclature heparin-binding growth factors is clearly to be preferred.

Frac 100 fraction collector. All chromatograms were obtained out at ambient temperature (*ca.* 18°C). Bulk solvents and mobile phases were filtered through 0.5-μm filters (Millipore, Bedford, MA, U.S.A.) and degassed by sonication. The flow-rate was 1.0 ml/min. All samples were loaded onto the reversed-phase columns directly as the effluent from the heparin-Sepharose affinity chromatographic step through a modified Waters U6K injector. The eluted fractions were collected as 500-μl aliquots in polypropylene tubes, and the acetonitrile–trifluoroacetic acid immediately removed under vacuum using a Savant Speedivac.

The following methods are representative of the procedures employed for the tryptic digestion and RP-HPLC mapping of the bovine HBGFs. Freshly isolated HBGF (2 μmoles) was dissolved in 100 μl of 400 m*M* Tris–HCl (pH 8.0), 4 m*M* EDTA, 6 *M* guanidine hydrochloride. The solution was incubated with vigorous shaking at 37°C for 30 min. Dithiothreitol was added in a 1:1 (w/w) ratio, the reaction mixture was flushed with nitrogen and then incubated for 3 h at 37°C with vigorous shaking. Iodoacetic acid (Fluka) in a 1.9 excess (w/w) to dithiothreitol, was added as a solution in 1.0 *M* Tris–HCl (pH 8.6). Following flushing with nitrogen, the mixture was incubated for 15 min at room temperature with shaking. The reaction was then quenched with 5 μl of 2-mercaptoethanol. The alkylated protein was separated from other contaminants by RP-HPLC on a Bakerbond C_4 (5 cm × 4.6 mm) column. The reaction mixture from the reductive alkylation was loaded directly onto the column and then eluted with 60 ml of 0.1% trifluoroacetic acid (TFA) at a rate of 1 ml/min, followed by elution with a 20-min, 0.1% TFA, 0–50% acetonitrile linear gradient. The carboxymethylated factor was eluted at *ca.* 45% acetonitrile concentration and was collected in tubes containing SDS (100 μg in 100 μl) to enhance recoveries. The appropriate fractions were pooled and concentrated to 150 μl with a Speedivac concentrator (Savant). TPCK-Treated trypsin (Wortington), dissolved in 1 m*M* hydrochloric acid, was added at a ratio of 1:100 (w/w) to protein; nine volumes of HPLC-grade methanol were added, and the mixture was kept at −20°C overnight. The mixture was then centrifuged for 25 min on a Microfuge (Beckman), and the supernatant was removed. The precipitate was resuspended in 100 μl of 100 m*M* ammonium bicarbonate, 2 m*M* calcium chloride, together with trypsin at a ratio of 1:100 (w/w) to protein. Following overnight incubation at room temperature, the digestion fragments were separated by RP-HPLC with a Bakerbond C_4 column. Elution was performed over 100 min at 1 ml/min with a 100 m*M* ammonium bicarbonate, 0–30% acetonitrile linear gradient and for a further 20 min, during which time the acetonitrile concentration was increased to 50%. The homogeneity of each of the tryptic peptide peaks was determined by rechromatography with a 0.1% TFA, 0–50% acetonitrile linear gradient over 60 min. Sequences of tryptic peptides were determined on an Applied Biosystem Model 470A sequencer with subsequent analysis of the phenylthiohydantoin (PTH)-amino acids by RP-HPLC using a Zorbax ODS column (DuPont, Wilmington, DE, U.S.A.) with a 50 m*M* sodium acetate–methanol (0–75%) gradient elution system at a flow-rate of 1.5 ml/min.

Mitogenic assay

The [^{3}H]thymidine uptake assay with Balb/C 3T3 fibroblasts, dispersed as monolayer cultures at a density of *ca.* $3 \cdot 10^3$ cells/100 μl in Dulbecco minimum medium, containing 5% foetal calf serum, was employed. Incorporation of radio-

activity was determined with a Packard Tricarb scintillation counter, following cell disruption with SDS and precipitation of the cell contents with 10% trichloroacetic acid.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Analytical SDS-PAGE according to the method of Laemmli[8] in vertical 12.5% acrylamide gels was used in the presence/absence of β-mercaptoethanol to monitor the purification of the HBGF. Polypeptides were visualised by the silver staining method of Wray *et al.*[9].

RESULTS AND DISCUSSION

HPLC, and in particular RP-HPLC with microparticulate chemically modified *n*-alkylsilica stationary phases, has proved over the past several years to be a very powerful tool for the resolution and purification of bioactive peptides and polypeptides in biological extracts. These rapid techniques have allowed the micropreparative isolation to homogeneity of a large array of novel peptide-releasing factors, and polypeptide/protein hormones involved in endocrine/exocrine biological pathways. Similar methods are proving very useful in studies on the purification of paracrine and autocrine polypeptide factors as well as in studies on the biosynthesis and post-translational processing of individual precursor proteins.

It has been known[10] for over 40 years that extracts of fowl and rat brain tissue would promote the growth of cultured fibroblasts. This activity, parenthetically called fibroblast growth factor, consists of a group of polypeptide mitogens, some of which exhibit pronounced affinity for heparin. Although initial attempts to define the chemical nature of these polypeptide mitogens produced conflicting results, recent studies in this and other laboratories have led to the development of new procedures for the purification of these factors. In particular, the introduction of heparin-Sepharose and biomimetic dye affinity steps[4,11–14], considerably simplified the purification over the earlier, time-consuming conventional ion-exchange procedures. Typically, the mitogenic activity at the heparin-Sepharose stage is eluted with a 0.8–2.0 *M* sodium chloride gradient. Under these conditions, both the α- and β-form of these polypeptide mitogens (α-HBGF and β-HBGF), can be separated. Although the α- and β-HBGF share some amino acid sequence homology[4,5,12], and similar cell proliferative properties, SDS-PAGE results indicate apparent differences in molecular weights. It is thus likely that separate genes code for α- and β-HBGF.

In the course of our purification studies[4,14] it became evident from the results of Western transfer immunoblotting experiments and other techniques, that other immunoreactive forms of α- and β-HBGF were present in both the original biological extracts and at different stages of purification. Furthermore, indications from amino-terminal microsequencing experiments pointed to the possibility that α- and β-HBGF occurred as different truncated forms, largely unique to the original source of tissue. Although analytical SDS-PAGE or high-resolution size-exclusion chromatography were unable to resolve these microheterogeneous forms, separation of the different molecular species could be readily achieved by RP-HPLC. Typical of these results are the chromatograms shown in Figs. 1 and 2. Fig. 1 shows the chromatographic profile and location of major mitogenic zones when the 0.8–1.0 *M* so-

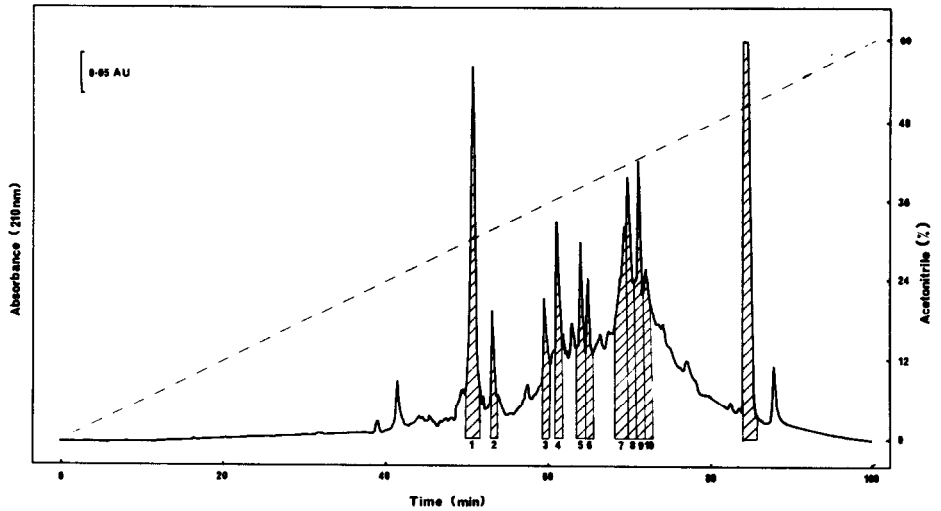

Fig. 1. Chromatographic profile obtained following RP-HPLC of the 0.8–1.0 *M* sodium chloride polypeptide components from the heparin-Sepharose fractionation of bovine brain polypeptide mitogens. Chromatographic conditions: column, Bakerbond C_4 (15 × 0.46 cm I.D.); flow-rate, 1 ml/min; mobile phase, 0.1% TFA (solvent A) to 60% aq. acetonitrile–0.092% TFA (solvent B), gradient time 90 min with a further 30 min at 100% solvent B. Shaded areas represent bioactive zones collected for subsequent rechromatography on the same stationary phase under different gradient conditions.

dium chloride HBGF component from heparin-Sepharose chromatography was rechromatographed on a Bakerbond C_4 column with a 0–60% acetonitrile gradient, containing 0.1% TFA. Fig. 2 shows a similar chromatogram, corresponding to the 1.0–1.3 *M* sodium chloride HBGF component from the heparin-Sepharose chromatography step. When aliquots from the recovered chromatographic fractions were

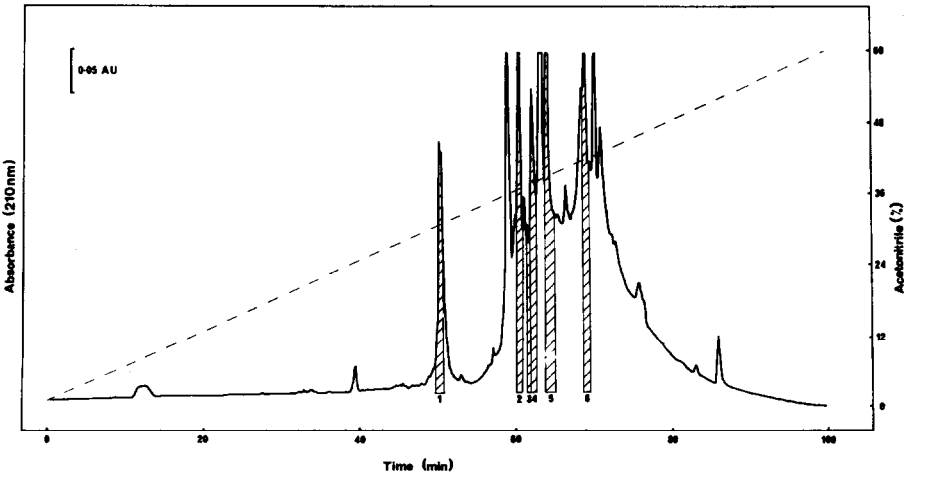

Fig. 2. Chromatographic profile obtained following RP-HPLC of the 1.0–1.3 *M* sodium chloride polypeptide components from the heparin-Sepharose step in the fractionation of bovine brain polypeptide mitogens. Chromatographic conditions as in the legend to Fig. 1.

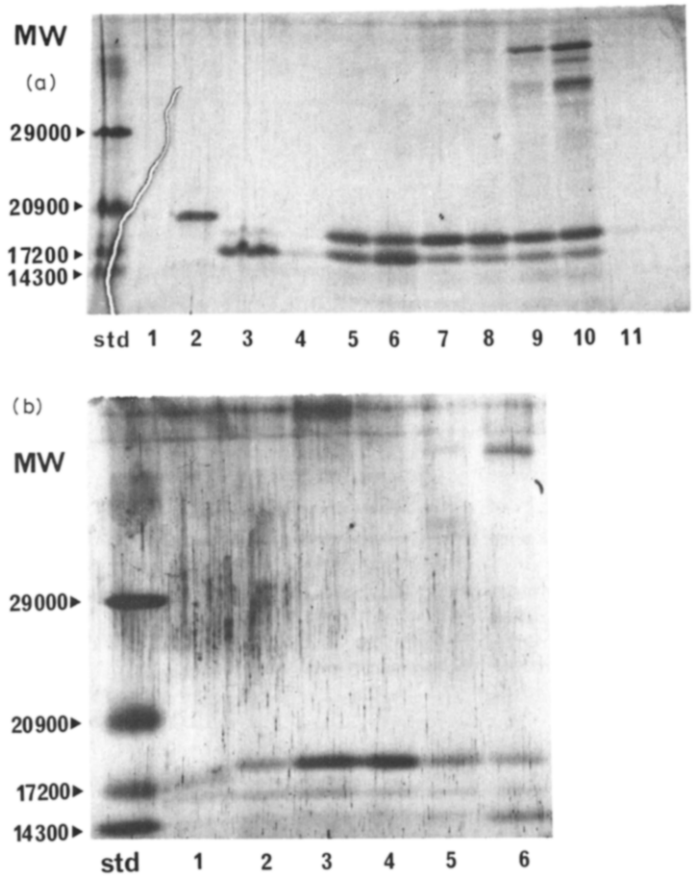

Fig. 3. PAGE analysis of chromatographic fractions derived from the RP-HPLC separations (a: Fig. 1; b: Fig. 2) of the HBGF. MW = Molecular weight. The lane assignments correspond to the shaded chromatographic zones shown in Figs. 1 and 2.

analysed by SDS-PAGE, the results (Fig. 3) clearly demonstrated that RP-HPLC allows further resolution of the mitogenic heparin-Sepharose polypeptide peak into five to ten fractions, which correspond to two polypeptides with apparent molecular weights of either 14000 or 17500. Rechromatography of individual bioactive zones on the same stationary phase under modified gradient and eluent conditions allowed individual forms of α- and β-HBGF to be isolated as chromatographically and electrophoretically homogeneous components. The amino-terminal sequence characterisations of these different forms is currently under investigation. Typical of these studies are the data obtained for the β-HBGF-I form. The separation of the tryptic peptides derived from the carboxymethylated polypeptide of this β-HBGF-I form, is shown in Fig. 4. The carboxymethylated β-HBGF-I polypeptide exhibits an amino-terminal sequence of Glu^{1}-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro^{10}-Gly-His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr^{20}... Although several peptides were eluted simultaneously, *i.e.* peptides Tyr-Leu-Ala-Met and [carboxymethyl]-Cys-Val-Thr-Asp--Glu-[CMC]-Cys-Phe-Phe-Phe-Glu, when 0.1% TFA was used as the ionic modifier

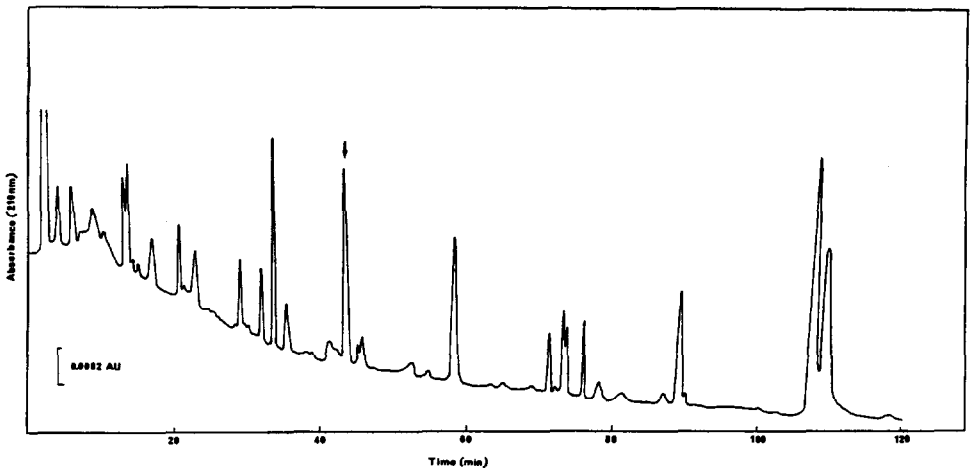

Fig. 4. RP-HPLC of the tryptic digest of the β-HBGF-I polypeptide. The chromatographic conditions were: column, Bakerbond C_4 (15 × 0.46 cm I.D.) flow-rate, 1.0 ml/min; mobile phase, 0.1% TFA (solvent A) to 60% aq. acetonitrile–0.1% TFA (solvent B), grdient time 90 min, followed by 30 min at 100% solvent B. The coincident elution position of the tryptic peptides CVTDECFFFE and YLAM is indicated by the arrow. Rechromatography of this zone with a 100 m*M* ammonium bicarbonate 0–50% acetonitrile linear gradient over 60 min, resulted in resolution of these two tryptic peptides.

of the first eluent system, the combination of 100 m*M* ammonium bicarbonate as the first eluent system and 0.1% TFA as the second elutent system (both based on a 0–50% acetonitrile gradient) permitted all the tryptic peptides to be resolved. Structional characterisation by microsequencing techniques indicated that all these HBGF forms are related and that the 14-kilodalton forms are derived as truncated products of the 17.5-kilodalton forms[15].

In summary, this study confirms that HBGF, as obtained by heparin-Sepharose affinity chromatography of bovine brain extracts, exists in at least two molecular-weight forms, which can be distinguished and further resolved by RP-HPLC. The biological roles of these truncated forms, and of the higher-molecular-weight species, which exhibit immunoreactivity with specific antisera raised against the 17.5-kilodalton form of HBGF, are of great physiological interest, particularly since tissue-specific processing of the precursor protein(s) is evident from tissue localisation studies[14]. These and other aspects of the structure of the truncated HBGF are currently under investigation.

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