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Short Communication

Purification of growth hormones by reversed-phase high-performance liquid chromatography

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ABSTRACT

Reversed-phase high-performance liquid chromatography (HPLC) on a column of Radial-Pak C_{18} cartridge was utilized for the purification of a variety of growth hormone (GH) proteins from mammalian, avian, amphibian and fish pituitary glands. Recovery of GH from pituitary glands of up to 0.43% of total protein was obtained with a high degree of homogeneity as revealed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The HPLC-purified GHs show reactions of identity or near identity by immunodiffusion studies on agar gel. This method offers a convenient and rapid purification of vertebrate GH on an analytical or preparative scale.

INTRODUCTION

Reversed-phase HPLC possesses significant resolving power and sensitivity for the analysis of proteins. The method is particularly well suited to the isolation of proteins on an analytical or preparative scale. Spitsberg [1], Kobayashi *et al.* [2] and Farmer *et al.* [3] have reported the purification of growth hormones (GHs) by column chromatography. However, the methods are often time-consuming and not well suited to purification at the submilligram level. Although HPLC has been used for the isolation of GH [2], it usually follows one or more steps of column chromatography.

We report here the development of a new system for the purification of pituitary GHs using one-step reversed-phase HPLC.

EXPERIMENTAL

Pituitary extracts

Fresh pituitary glands were obtained from a local market. They were homogenized with 50 m*M* ammonium bicarbonate (pH 8.2) containing 1 m*M* phenyl methylsulphonyl fluoride (PMSF)

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at 4°C. The homogenate was centrifuged at 12 000 *g* for 5 min and residues re-extracted several times.

Chromatographic apparatus and procedures

Separations were accomplished with a system consisting of a Waters RCM-100 radial compression module and C_{18} cartridges (C_{18}-bonded silica, 10 μm particle size, 10 mm × 8 mm I.D.). Pituitary extracts were injected onto the appropriate column. Samples were then eluted with a linear gradient of two solvents: 0.1% trifluoroacetic acid (TFA)–water (solvent A) and 0.1% TFA–acetonitrile (solvent B). The column was developed with a 40-min gradient from 30% B solvent to 70% B solvent at a flow-rate of 1.0 ml/min. All chromatographic procedures were performed at room temperature. Columns were equilibrated with the starting solvent prior to sample application. At the end of each chromatographic run, columns were washed with 100% solvent B for 15 min and re-equilibrated. Proteins in the eluate were monitored at 280 nm with a UV detector and collected manually. Eluates were desiccated to remove organic solvent and lyophilized.

Electrophoresis

Analysis of protein fractions collected from HPLC was accomplished by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% acrylamide gels containing 0.1% SDS by the method of Laemmli [4]. Gels were stained for protein with Coomassie brilliant blue R-250.

Protein determination and amino acid analysis

The amino acid composition of protein samples was determined following hydrolysis in 6 *M* hydrochloric acid containing 10% TFA and 0.1% phenol for 30 min at 158°C in evacuated tubes. The hydrolysate was dried and treated by the method of Chang and Liu [5] for amino acid analysis. Protein content was calculated from the actual amounts of constituent amino acids.

Amino acid sequencing

The amino acid sequence was determined in a 477A pulse-liquid phase protein sequencer (Applied Biosystems, Foster City, CA, USA).

Immunological characterization

Monkey anti-rat GH was used to check the immunological reaction of the purified GHs. This was done in an agar plate according to Ouchterlony [6].

RESULTS AND DISCUSSION

The elution profiles of pituitary extracts from a Radial-Pak C_{18} cartridge are shown in Fig. 1. In order to obtain better resolution, elution conditions for duck and carp GHs by reversed-phase HPLC were modified as shown in Fig. 1F and G. Fractions from individual peaks were collected and examined. In most cases the GH was eluted by 60–70% acetonitrile in the peak with the longest retention time. The GHs were identified by SDS-PAGE (Fig. 2), immunoreaction with GH-specific antiserum (Fig. 3) and partial amino acid sequencing in some cases [7–9]. An additional band of 24 kDa was usually seen on SDS-PAGE of bullfrog GH, which was probably due to contamination by prolactin. In this case the GH fraction can be purified by one more cycle of HPLC [10]. On the other hand, prolactins of some species can also be obtained at the same time as GH purification. The GH-containing fraction should be carefully collected in order to avoid contamination of neighbouring peaks. When necessary, the collected GH fraction can be purified with one more cycle.

The yields of GHs purified from various species are summarized in Table I. The average recovery was around 0.43% of total protein. The GHs purified from the reversed-phase HPLC are pure enough for further studies such as amino acid composition analysis and amino acid sequencing [7–9].

In our procedure, the GHs are effectively extracted by 50 m*M* ammonium bicarbonate (pH 8.2) at 4°C rather than by acid acetone or other salts. The pituitary extract is directly applied to

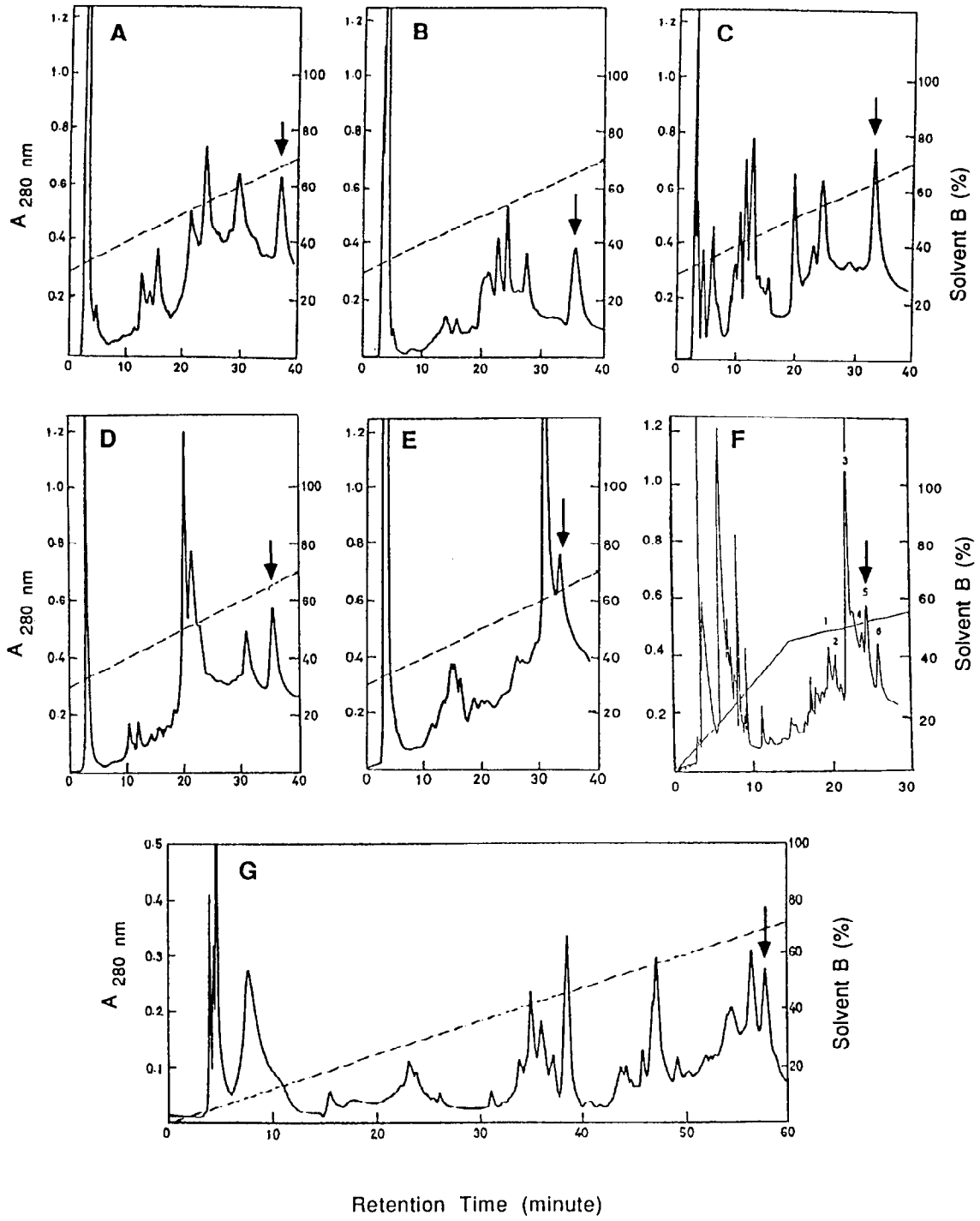

Fig. 1. Reversed-phase HPLC of pituitary extracts of (A) sheep, (B) goat, (C) pig, (D) rat, (E) bullfrog, (F) duck and (G) carp. Solvent A: 0.1% trifluoroacetic acid (TFA)–water. Solvent B: 0.1% TFA–acetonitrile. Arrows indicate the peaks of GH.

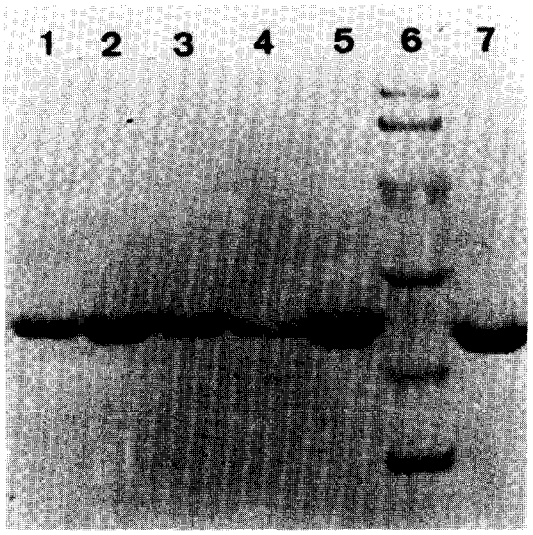

Fig. 2. SDS-PAGE of GHs purified by reversed-phase HPLC. Lanes: 1 = rat; 2 and 3 = pig; 4 = goat; 5 = sheep; 6 = molecular weight markers: phosphorylase b (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa); lactalbumin (14.4 kDa); 7 = bullfrog.

the reversed-phase HPLC system in order to avoid the loss of GH and to simplify the procedure. We did not measure the biological activity of the purified GH. However, in view of studies on fish and amphibian GH [2,11], the use of HPLC for the purification of bioactive GHs indicates that a short exposure to HPLC conditions does not abolish biological activity. It seems highly likely that the GH purified by this method will also be active.

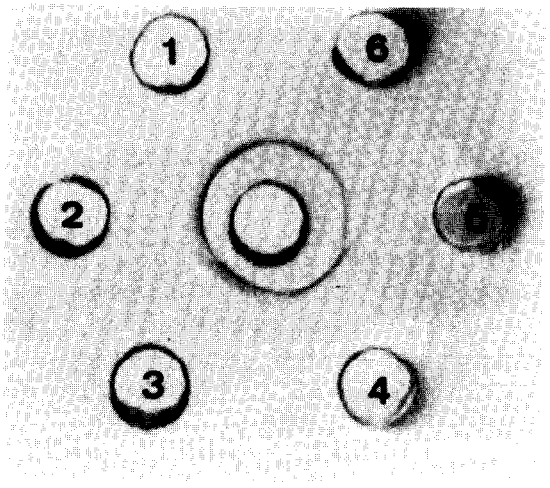

Fig. 3. Immunoprecipitation reactions of purified GHs against monkey antiserum to rat GH (centre well). 1 = Sheep; 2 = goat; 3 and 6 = pig; 4 = bullfrog; 5 = rat.

TABLE I

YIELDS OF GROWTH HORMONE FROM VARIOUS SPECIES

Species	Recovered GH from 2 mg of total protein (μg)	Percentage
Pig	9.64	0.48
Sheep	9.80	0.49
Goat	9.40	0.47
Rat	7.70	0.39
Bullfrog	6.15	0.31

With its simplicity and high resolving power, reversed-phase HPLC is suitable for rapid purification of GHs from small amounts of pituitary glands with minimal time and effort. Our results demonstrate that the GHs obtained by this method are homogeneous as determined by SDS-PAGE analysis and are immunoreactive. The protocols described herein may also be used for the analysis and purification of prolactin [12].

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