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PARTIAL PURIFICATION OF A SPECIFIC INHIBITOR OF THE INSULIN-LIKE GROWTH FACTORS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ADRIEN D. KUFFER and ADRIAN C. HERINGTON*

Medical Research Centre, Prince Henry's Hospital, St. Kilda Road, Melbourne, 3004 (Australia)

SUMMARY

We report here preliminary data using reversed-phase high-performance liquid chromatography for the purification of a specific inhibitor (a molecular weight 16,000—18,000 protein) of the insulin-like growth factor (IGF) or somatomedin family. Crude inhibitor prepared from Cohn fraction IV-1 of human serum was first partially purified using an IGF/CH-Sepharose 4B affinity column. Following elution of the bound inhibitor and resuspension in 0.1% aqueous trifluoroacetic acid (mobile phase A), it was injected (100 μl; 2.0 mg protein) onto a Brownlee Aquapore RP-300 column. Application of a linear gradient from 0% to 100% mobile phase B (45% isopropanol—0.1% trifluoroacetic acid) resulted in elution of two peaks of inhibitor activity between 31% and 34% isopropanol associated with a major homogeneous protein peak and a minor heterogeneous protein peak. No inhibitor was recovered when an acetonitrile gradient was used instead of isopropanol, indicating that the inhibitor is very hydrophobic. These data suggest that high-performance liquid chromatography offers a simple procedure for the potential purification of IGF inhibitor(s) from normal human serum.

INTRODUCTION

The insulin-like growth factors (IGF) or somatomedins (Sm) [IGF-I/SmC, IGF-II, SmA] are a family of circulating polypeptide growth factors with a marked, but not absolute, dependence on serum levels of growth hormone (GH) [1]. IGF-I/SmC has been shown to mediate the actions of GH on skeletal growth of hypophysectomized rats [2]. A variety of specific and non-specific inhibitors of the IGFs have also been reported in several severe catabolic states in humans and experimental animals [1]. Recently, we have identified, for the first time in normal human serum, a quite specific inhibitor of many of the actions of the IGFs [3] (Table I). This suggests that under normal physiological circumstances normal growth may well be dependent on an adequate balance

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TABLE I

PHYSICOCHEMICAL AND BIOLOGICAL CHARACTERISTICS OF IGF INHIBITOR

(1)	Proteinaceous, M_r approx. 18,000, p*I* 4.0—4.4 Acid-stable, heat-labile
(2)	Causes competitive inhibition of (a) IGF cartilage sulphation activity (b) IGF adipocyte non-suppressible insulin-like activity (c) IGF binding to placental and adipocyte receptors
(3)	Mechanism of action via direct interaction (binding) with IGF
(4)	Effects are dose-dependent and specific for the IGF/Sm family of growth factors

between these positive (IGF/Sm) and negative (inhibitor) factors. In order to substantiate this hypothesis one requires better characterization and identification of the inhibitor(s) together with the establishment of a specific and quantitative assay. Thus, we have attempted to further purify this substance from fractions of normal human serum and report here the results of our initial studies using reversed-phase high-performance liquid chromatographic (HPLC) techniques.

EXPERIMENTAL

Materials

Sephadex G-75 and CH-Sepharose 4B were from Pharmacia (Upsala, Sweden). Bio-Gel P-30 was from Bio-Rad (Richmond, CA, U.S.A.). Trifluoroacetic acid (TFA) was from BDH (Poole, England) and HPLC-grade isopropanol was from Ajax Chemicals (Sydney, Australia). Ultrafiltration membranes were from Amicon (Lexington, MA, U.S.A.).

Preparation of a crude inhibitor fraction

A crude IGF inhibitor fraction was prepared from Cohn fraction IV-1 of human serum as reported previously [3]. This involved initial ion-exchange chromatography on SP-Sephadex C-25, with application at pH 5.5 followed by sequential batch-wise elutions at pH 5.5, 6.5 and 9.7. This final elution fraction, which contained both the active IGF species as well as the inhibitor, was ultrafiltered (Amicon UM-2 membranes), dialysed and lyophilized. This fraction was redissolved in 1% formic acid (pH 2.3) prior to sequential gel chromatography under acid conditions on Sephadex G-75 (120 × 4.2 cm) and Bio-Gel P-30 (48 × 2.3 cm) columns. This latter chromatography step separates the IGFs (M_r approx. 7500; K_{av} 0.4—0.9) from the inhibitor (M_r 16,000—18,000; K_{av} 0.1—0.4) which was dialysed and lyophilized.

Affinity chromatography of inhibitor

Previous studies (unpublished) had indicated that the mechanism of action of the inhibitor was via a direct interaction with the IGFs. Thus, this property was utilized to further purify the inhibitor on an IGF/CH-Sepharose 4B affinity

column. A mixed preparation of IGFs was prepared by the method of Svoboda et al. [4] and separated from inhibitor activity by Bio-Gel P-30 chromatography in 1% formic acid as described above. The IGF was covalently linked to CH-Sepharose 4B according to the manufacturer's recommendations. The Bio-Gel P-30 inhibitor fraction was resuspended in Krebs—Henseleit bicarbonate buffer pH 7.4 (25 m*M*) and mixed with the IGF/CH-Sepharose. The mixture was placed in a glass column, stoppered and rotated end-over-end at 4°C for 16 h to allow IGF—inhibitor interaction to reach equilibrium. The column was then attached to a fraction collector and unbound proteins eluted with Krebs—Henseleit buffer until $A_{280\,nm}$ reached baseline (30 × 1-ml fractions). Bound proteins were then eluted with 1% formic acid (20 × 1-ml fractions). Acid fractions 3—8 contained inhibitor activity and were pooled, dialysed and lyophilized.

High-performance liquid chromatography

The lyophilized inhibitor was redissolved in 0.1% (v/v) aqueous TFA and an aliquot (100 μl; 2—3 mg protein) was injected onto a Brownlee Aquapore RP-300 (10 μm) column (25 cm × 4.5 mm) (Brownlee Labs, Santa Clara, CA, U.S.A.) attached to a Waters liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.). The system was equipped with dual Model 6000A solvent delivery systems, a Model 660 solvent programmer, a Model 441 absorbance detector, a Gilson (Villiers le Bel, France) Microcol TDC80 fraction collector and an Omniscribe (Houston Instruments, Austin, TX, U.S.A.) dual-pen recorder. Mobile phase A was a 0.1% (v/v) aqueous TFA solution. A linear gradient from 0% to 100% mobile phase B (45% isopropanol—0.1% TFA) was applied over 60 min at a flow-rate of 0.7 ml/min. Fractions (1 min) were collected, the protein elution profile was monitored at $A_{215\,nm}$ and the samples then dried in vacuo at 37°C and resuspended in Krebs—Henseleit buffer prior to assay.

Assay for inhibitor activity

Inhibitor activity was assessed by the ability of samples to inhibit IGF activity in a standard IGF/Sm bioassay system. This bioassay measures the stimulation by IGF of [^{14}C]glucose incorporation into [^{14}C]lipid by isolated rat adipocytes [5]. The effect of inhibitor on IGF activity in this system is dose-dependent and specific [3] but is essentially a qualitative rather than quantitative measurement of inhibitor activity. To facilitate the assay of many HPLC fractions, only one dose level (in duplicate) of each fraction was assayed against a single dose of a standard crude preparation of IGF activity (i.e. equivalent to 20%, v/v, of an acid—ethanol extract of a pool of normal human serum [5]). Inhibitor activity has been expressed as the percentage inhibition of the stimulation by that standard dose of IGF.

RESULTS AND DISCUSSION

The HPLC protein elution profile, plotted against the fraction number and percentage isopropanol is shown in Fig. 1A. Fig. 1B illustrates the IGF inhibitor activity profile. The inhibitory activity was eluted from the HPLC column as two peaks of activity between 31% and 34% isopropanol and

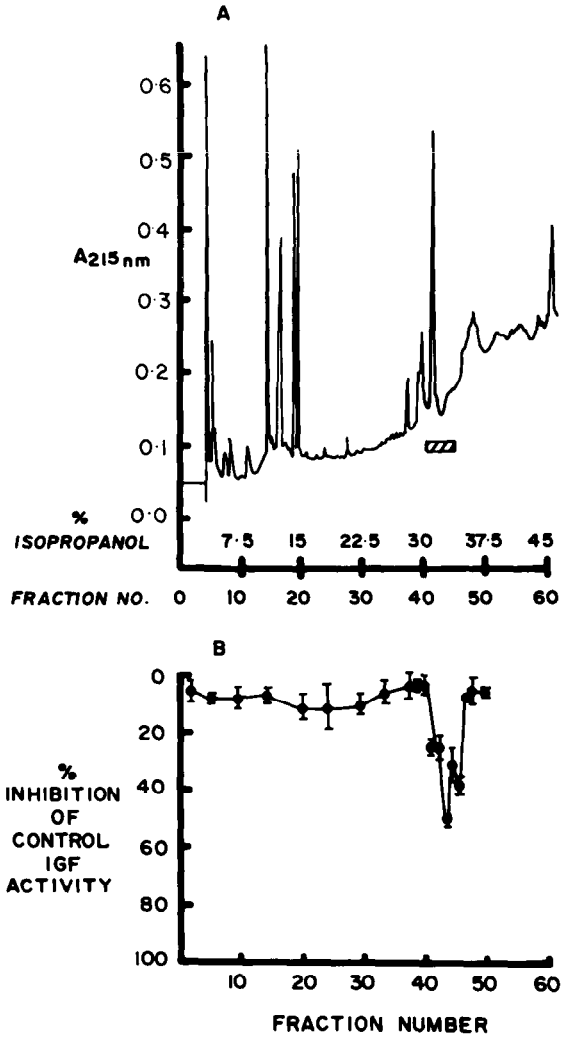

Fig. 1. HPLC of IGF affinity purified inhibitor. (A) Protein elution profile ($A_{215\,nm}$) with ▨ representing inhibitor activity profile as shown in B. Inhibitor activity was measured in pools of five consecutive fractions across the profile except for fractions 41—45 which were assayed individually. Each vertical bar represents the range of the duplicate determinations on each fraction (or pool). HPLC was carried out as described in the Experimental section.

corresponded to a major, apparently homogeneous, protein peak and a minor heterogeneous peak in Fig. 1A. Since the method of assay currently used is not entirely quantitative it is difficult to adequately assess recoveries. However, in other similar experiments (data not shown, but for example see Fig. 2B) the sum of the inhibitor activity present in HPLC fractions was equivalent to that of the activity of the aliquot of the original inhibitor sample applied to the HPLC column, suggesting a substantial recovery.

An IGF inhibitor can also be isolated from Cohn fraction IV-1 of human serum by methods other than that described above in the Experimental section, providing one includes the Bio-Gel P-30 acid chromatography. This step separates the inhibitor from the stimulatory IGF species and has been used to

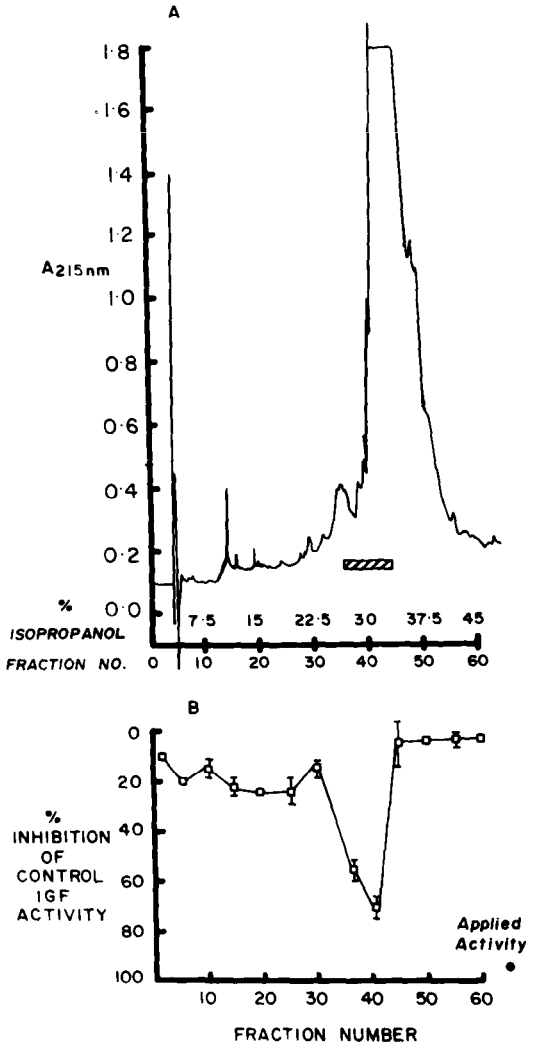

Fig. 2. HPLC of inhibitor without prior IGF affinity purification. (A) Protein elution profile ($A_{215\ nm}$) with ▨ representing inhibitor activity profile as shown in B. Inhibitor activity was measured in pools of five consecutive fractions across the profile. HPLC was carried out as described in the Experimental section.

isolate inhibitor from the recently reported IGF purification method of Svoboda et al. [4]. This inhibitor preparation was applied to the same HPLC system described above, although without prior IGF affinity purification. Because this was a much cruder preparation, the chromatogram (Fig. 2A) revealed that most of the protein ran as a large, broad peak between 30% and 40% isopropanol. However, the inhibitor activity was eluted in a very similar position as that previously described (Fig. 1) although it was recovered as a broader peak with activity spanning 27—34% (Fig. 2B). The activity was associated with the leading edge of the large heterogeneous protein peak. This data suggests that the IGF inhibitors isolated by the two methods [3, 4] are similar if not identical.

The reversed-phase HPLC conditions outlined here were achieved after studies using a variety of ion-pairing reagents (TFA, triethanolamine phosphate, ammonium hydrogen carbonate) and column packings (C_8, C_{18}, CN) together with acetonitrile gradients up to concentrations of 80%. In none of these experiments was inhibitor activity recoverable, despite the fact that inhibitor is stable to such acetonitrile concentrations (unpublished data). This would suggest that the inhibitor is quite hydrophobic. The IGF affinity column has also been used to isolate inhibitor(s) of IGF activity from other sources (e.g. directly from serum; liver extracts). However, the HPLC conditions used above did not result in recovery of inhibitory activity and other conditions are being examined for the purification of these particular species.

The significance of the presence in serum of such a specific inhibitor(s) of the IGF/Sm family is currently uncertain. The data presented here, however, do indicate that, together with IGF affinity purification, HPLC offers a simple procedure for the potential purification of this protein and should provide sufficient material to allow both the development of a direct and quantitative assay (e.g. a radioimmunoassay) for the inhibitor, and a study of its potential physiological role in modulating IGF/Sm stimulation of growth.

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