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Purification and characterization of three molecular forms of insulin-like growth factor II from human Cohn paste IV

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ABSTRACT

The somatomedins or insulin-like growth factors (IGFs) are a family of peptides present in human serum. They are bound to specific carrier proteins and are thought to mediate growth-promoting actions of human growth hormone Starting from Cohn fraction IV of human plasma, we describe here a rapid and highly efficient procedure for the purification to homogeneity, in addition to IGF I, of three forms of insulin-like growth factor II· IGF IIA (10–12 kDa), IGF IIB (the "classical" 7 5 kDa IGF II) and IGF IIC, identified as the IGF II variant of Jansen by fast-atom bombardment mass spectrometry The procedure is based on ion-exchange chromatography and gel permeation chromatography on Biogel P10 As judged by specific radioimmunoassay methods for IGF I and IGF II, one of the most striking advantages of this process at this stage is the yield of IGF I not contaminated by 7 5 kDa IGF II Isoelectric focusing or chromatofocusing, which require affinity chromatography to separate proteins from the polybuffers, are not necessary in this procedure Final purification was directly achieved by preparative, followed by analytical high-performance liquid chromatography The N-terminal sequence of peptide IGF IIB (39 amino acids) and peptide IGF I (29 amino acids) showed total homology with those previously described by Rinderknecht and Humbel [*FEBS Lett* , 89 (1978) 283] The final yields of purified human IGF I and IGF IIB were 15 and 25 μg, respectively, from 1 l of serum. All peptides interact with specific receptors on human lymphocytes and red blood cells, and are biologically active (stimulation of ^{35}S uptake, increasing [^{3}H]thymidine incorporation in human and chick emryo fibroblasts)

INTRODUCTION

The somatomedins [1] or insulin-like growth factors (IGFs) are polypeptides from human serum with growth-promoting effects *in vitro* [2] and *in vivo* [3].

IGF I identifical with somatomedin C [4] and IGF II were first purified and characterized from human plasma [5–7]. The primary structures of the prohor-

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mone forms of IGF I and IGF II have been deduced from their cDNA sequence [8–10]. IGF II is a single-chain peptide of 67 amino acids, synthesized as a 180-amino acid precursor and enzymatically processed to its mature from [9,11].

Variant forms of both IGF I [12] and IGF II [13,14] have recently been identified. A variant prepro-IGF II–cDNA has been isolated from a human liver cDNA library by Jansen *et al.* [15], in which the codon for Ser-29 was replaced by a 12-bp fragment coding for a tetrapeptide Arg-Leu-Pro-Gly. This variant codes for a mature IGF II of 70 amino acids and a precursor of 183 amino acids.

Zumstein *et al.* [16], without presenting data, stated that they had identified this variant in human plasma. They also isolated a 10-kDa variant of pro-IGF II that contained a substitution of Cys-Gly-Asp for Ser-33 as well as a carboxyl terminal extension of 21 residues [16].

The aim of the present study was to isolate and characterize multiple molecular forms of this peptide family from human serum. Samples at each stage of the purification were monitored both by radio-immunoassay (RIA) for IGF I and II and for sulphation activity in chick embryo cartilages and mitogenic activity in chick embryo fibroblasts.

EXPERIMENTAL

Assay

Immunoactivity. Specific RIAs for IGF I and IGF II were performed as previously described [17]. All results were corrected for cross-reaction.

Bioassay. Sulphatation activity was assayed at each step during the purification scheme, by measurement of the stimulation of the uptake of [^{35}S]sulphate in pelvic leaflets of chick embryos as described by Hall [18] and modified by Heulin *et al.* [19]. Samples were compared with a pool of normal adult human serum, defined as 1 unit of somatomedin per ml, at three doses (eight replicates per dose).

Crude material

A batch of 3 kg of Cohn paste IV (equivalent to 100 l of plasma) was used. Crude peptide material was extracted according to Svoboda *et al.* [20] in 30 l of 2.0 *M* acetic acid–0.075 *M* NaCl (pH 2.8). A large amount of insoluble protein was removed by centrifugation.

The cloudy extract was adsorbed overnight on 120 g of SP Sephadex C-25 (Pharmacia) resin pre-equilibrated in the same buffer as above. A washing step of 15 min with 2 l of 2.0 *M* acetic acid–0.4 *M* NaCl (pH 2.8) was followed by batch elution of the bound material with a pH step gradient in 0.2 *M* ammonium acetate–0.2 *M* NaCl buffer, at pH 5 (eluate B), pH 6 (eluate C) and pH 9 (eluate D) for 3 h each; the pH was adjusted with concentrated NH_4OH.

These eluates were immediately desalted on a 90 cm × 5 cm I.D. column of Tris GF 05 (IBF, France) in 1.0 *M* acetic acid at a flow-rate of 200 ml/h, lyophilized, resuspended and stored in 1.0 *M* acetic acid–0.05 m*M* ascorbate at +4°C.

Chromatography on Biogel P10

Gel permeation chromatography of each eluate was separately achieved on a 85 cm × 2.5 cm I.D. column of Biogel P10 (200–400 mesh, Biorad) equilibrated in 1 *M* acetic acid, as previously described [21]. The flow-rate was 20 ml/h. Absorbance of the eluent was recorded at both 254 and 280 nm (Uvicord II, Pharmacia). Fractions of 5 ml were collected and assayed for IGF immunoactivity [17] and somatomedin bioactivity [19].

Large-scale HPLC

Reversed-phase HPLC was performed using a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph consisting of dual M 6000A pumps, an M 720 solvent programmer, an M 481 variable-wavelength detector connected to a Spectra-Physics SP 4270 integrator, a WISP 712B injector and a Foxy 2200 fraction collector (ISCO, Lincoln, NE, U.S.A.).

The separation was performed on a Vydac 218 TP 510 (250 mm × 10 mm I.D., particle size 5 μm, pore size 300 Å) column (The Separations Group, Hesperia, CA, U.S.A.) operated at a flow-rate of 2.0 ml/min. Mobile phase A was 10 m*M* HCl and 0.15 *M* NaCl (pH 2.1) (Suprapur, Merck, Darmstadt, F.R.G.). Mobile phase B was 10 m*M* HCl and 0.15 *M* NaCl (pH 2.1) in water, containing 70% (v/v) acetonitrile (Hypersolv, BDH, Poole, U.K.) The effluent was monitored at 220 nm.

IGF II-containing fractions from Biogel P10 were injected by pumping directly through the import of pump A. After 20 min, the column was eluted with a linear gradient from 0 to 100% B over 85 min.

Small-scale HPLC

Further purification was carried out on the active material from the large-scale column. The equipment used was as described above, except that the column was a Vydac 218 TP 54 (250 mm × 4.6 mm I.D., particle size 5 μm, pore size 300 Å) or/and a Vydac 214 TP 54 (250 mm × 4.6 mm I.D., particle size 5 μm, pore size 300 Å). Solvent A was 0.085% (v/v) trifluoroacetic acid (TFA) aqueous solution (sequanal grade, Pierce, Rockford, IL, U.S.A.). Solvent B was 0.085% TFA in water, containing 70% (v/v) acetonitrile (LiChrosolv, Merck). The flow-rate was 0.7 ml/min. All solvents were degassed by sparging with helium. Detection of peaks was by UV absorbance at 214 nm. Bioactive samples from the large column were diluted (1:2, v/v) in aqueous TFA solution, injected as above, and a linear gradient from 30 to 100% B was applied over 85 min.

Amino acid analysis

Amino acids were analysed with a Waters Pico Tag system. Purified peptides were hydrolysed for 24 h at 110°C in the vapour of 6 *M* HCl–2 m*M* phenol in vacuo. Hydrolysates were analysed after conversion into phenylthiocarbamoyl (PTC) amino acid derivatives by HPLC, as described in detail in the analysis operator's manual [22].

TABLE I

PURIFICATION OF HUMAN INSULIN-LIKE GROWTH FACTOR II

Data represent five analyses, N D , not determined

Analysed fraction[a]	Specific sulphation bioactivity[b] (U/mg of protein)	Purification (*x*-fold)	RIA IGF II (μg equivalent)	Purity (%)	Recovery[c] of protein (%)
Serum	0 016	1	600 ± 150[d]	–	100
Acetic acid extract of Cohn paste IV	0 054	3 2	N D	–	N D
SP-Sephadex C-25					
Eluates B + C + D	9 78	586			
Eluate D			135	0 6	23
Biogel P10					
Peak IIA	3 05	128	6 1	0 8	12.2[e]
Peak IIB	45 45	2 730	53	6 9	8 8
Preparative HPLC					
Peak IIA	N D	–	3 4	N D	6 8
Peak IIB	821.4	49 300	31	79 3[f]	5 2
Analytical HPLC					
Peptide IGF IIB	N.D.	–	16	96 4[f]	3
Peptide IGF IIC	N.D.	–	1 9	94 7[f]	

[a] For a detailed description, see Experimental

[b] One unit is defined as the amount of somatomedin bioactivity present in 1 ml normal adult human male serum

[c] Recovery per l of original plasma (3 kg Cohn paste IV derived from 100 l of plasma).

[d] As in ref. 17

[e] 1 ml of plasma contained *ca* 50 μg of big "IGF II" as in ref 16.

[f] Protein content determined by amino acid analysis

Sequence analysis

Stepwise Edman degradation of the unmodified, highly purified peptides was kindly performed by Dr. Denoroy (Service Central d'Analyse, Centre de Séquençage, CNRS, Vernaison, France), with a gas phase protein microsequenator (Model 470 A) from Applied Biosystems (Foster City, CA, U.S.A.). Polypeptides obtained by HPLC were applied directly to the sequenator. The phenylthiohydantoin (PTH) amino acids were identified according to Hunkapiller and Hood [23]. Sequence analysis was repeated twice and data were uniformly consistent

RESULTS

Isolation of IGFs/somatomedins

Table I summarizes the purification procedure, which was monitored both by biological assay and by specific IGF I and IGF II RIA.

The acetic acid extract of Cohn paste IV is well known to be a convenient starting material [24]. Recovery of bioactivity ranged from 30 to 45%, compared with normal serum With a pH step gradient in ammonium acetate buffer, eluted fractions (eluates B + C + D) from SP Sephadex C-25 have an apparent 586-fold increase in specific bioactivity with 23% recovery for immunological IGF II material in eluate D.

Further purification was achieved by gel permeation on Biogel P10 in 1 *M* acetic acid (Fig. 1) and yielded three major peaks Peak I (k_{av} 0 36–0.43) contained more than 80% of IGF I immunoactivity. In contrast, IGF II elutes in two regions: one at k_{av} 0.25–0.35, named peak IIA, contained 10–15% of total IGF II and is consistent with the 10 kDa IGF II component described by Zumstein *et al.* [16]; the other, at k_{av} 0.45–0.70 and named peak IIB, with 80% of total IGF II RIA activity, is probably similar to the 7 kDa IGF II peptide purified by Rinderknecht and Humbel [7].

All these fractions stimulated sulphate incorporation in chick embryo pelvic leaflets, with 44.3 [25] and 45 5 U/mg of protein as specific activity for peak I and peak IIB, respectively. Peak IIA, with 3.05 U/mg of protein, is contaminated by some inhibitors [26]. The recovery (less than 15%) of IGF II peptides is poor and varies from batch to batch (see Table II): it is *ca* five times lower than that for IGF I, although the concentration of IGF II in normal serum is three or four times higher than that of IGF I.

However, as shown in Table II, the most important point is that contamination by IGF II of peak I is less than 3%. Similarly, peak IIB contained less than 5% of RIA IGF I activity. Purification of these peptides on Biogel P10 is quite sufficient to undergo final purification directly by HPLC [25]. All procedures previously described have a chromatofocusing (or isoelectric focusing) step before HPLC. To overcome carrier–ampholyte–peptide interactions [27], bioactivity determination needed an ultimate step (better in immunoaffinity) to separate peptides fully from polybuffers.

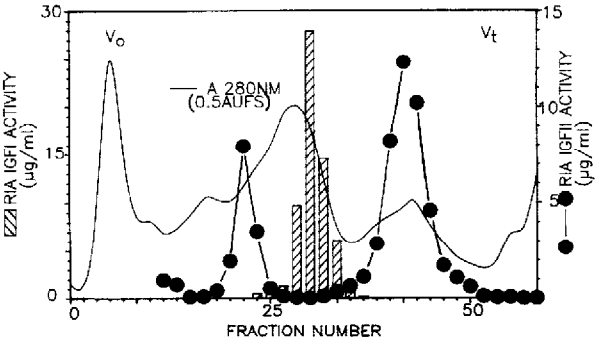

Fig. 1 Elution profile of proteins and immunoactivity from the Biogel P10 column. Eluate D from SP-Sephadex was applied to a 100 cm × 2 6 cm I.D. column equilibrated and eluted with 1 *M* acetic acid Fractions (5 ml) were collected every 15 min. The absorbance at 280 nm was monitored continuously Fractions (peak IIA and peak IIB) were used separately for further purification

As confirmed by Baxter and De Mellow [28], most IGF I eluted in eluate B and more in C, whereas IGF II eluted in eluate D, particularly at pH 9.0. Further purification of IGF II was achieved from the latter extracting buffer.

Peak IIA (fraction numbers 19–25) and peak IIB (fraction numbers 37–45, separately injected) were subjected to C_{18} HPLC on a preparative scale.

TABLE II

RESULTS FROM DIFFERENT BATCHES ON BIOGEL P10

Batch No	Yield per 3 kg of COHN paste IV (µg)		Evaluation
	RIA IGF I	RIA IGF II	
Biogel P10 8401			Recovery for IGF I
Peak IIA	98	330	Mean, 2125
Peak I	1930	50	S D., 291
Peak IIB	5	1455	Range, 1760–2480
Biogel P10 8402			Recovery for 8–10 kDa IGF II
Peak IIA	75	480	Mean, 345
Peak I	1761	210	S.D., 135
Peak IIB	14	5510	Range, 130–480
Biogel P10 8405			Recovery for 5–7 kDa IGF II
Peak IIA	256	130	Mean, 2650
Peak I	2480	15	S D , –
Peak IIB	85	662	Range, 660–5510
Biogel P10 8502			
Peak IIA	201	445	$\frac{\text{IGF II}}{\text{IGF I}}$ *ca* 1 2 $\frac{\text{IGF IIA}}{\text{IGF IIB}}$ *ca* 0 13
Peak I	2330	140	
Peak IIB	35	2960	

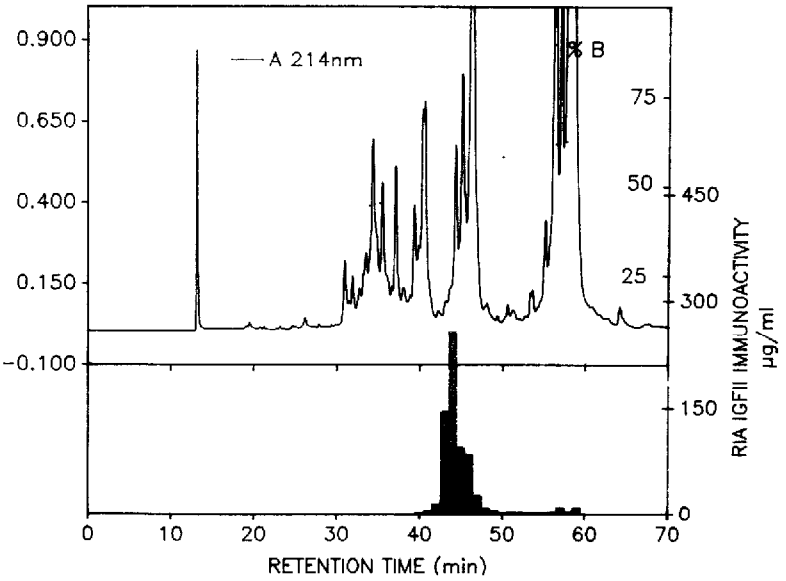

Fig 2 Reversed-phase HPLC separation of Biogel P10 peak IIA material Each fraction (tube No. 23, 5 ml) was applied to a Vydac 218 TP 510 column equilibrated with 10 m*M* HCl–0 15 *M* NaCl (pH 2.1) (mobile phase A). Elution was carried out at 2 ml/min by a linear increase in the concentration of mobile phase B (acetonitrile–mobile phase A, 70·30 v/v) as follows 0–5 min, 0–20% B, 5–23 min, 20–39% B; 23–38 min, 39–47% B, 38–48 min, 47–57% B, 48–58 min, 57–63% B, 58–70 min, 63–100% B, 10 min isocratically at 100% B; 80–85 min, 100–0% B for a total run time of 120 min Absorbance was monitored at 220 nm Fractions were collected every 0 5 min Immunoactivity was estimated by RIA IGF II (black area, lower panel).

The elution pattern of peak IIA (Fig. 2) showed a sharp peak of immunoactivity at 37.2% acetonitrile (44.24 min). Complete characterization of this peptide is under investigation. The distribution of eluted protein of peak IIB is presented in Fig. 3 The first peak, containing the majority of RIA activity, eluted at 37.3% acetonitrile (44.35 min) and is named peptide IGF IIB, with a specific bioactivity of 821.4 U/mg of protein. The second peak of competing activity, named peptide IGF IIC, eluted at 38.3% acetonitrile (45.72 min) and varied from batch to batch (5–17 area % of IGF II immunoactivity). Active fractions were diluted with two volumes of aqueous 0.08% TFA (final acetonitrile concentration 12%) and injected separately through the solvent line of pump A on an analytical scale, using the conditions described in legend of Fig. 4. Peptide IGF IIB, eluted at 32.85% acetonitrile (Fig. 4), was 96.4% pure after two reinjections; peptide IGF IIC was more than 94% pure (data not shown) after one reinjection and eluted at 32.92% acetonitrile.

All these different molecular forms might be the result of proteolytic degradation during extraction or during cellular processing. Their biological significance is currently being evaluated.

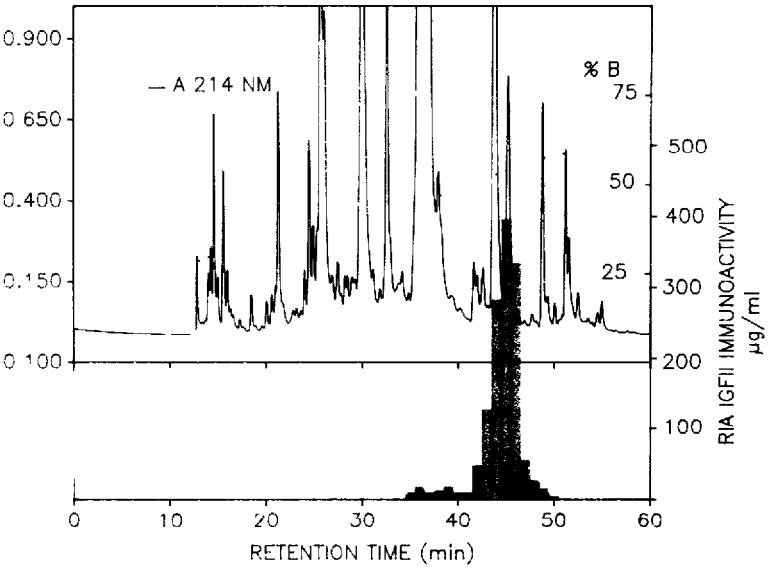

Fig. 3 Reversed-phase HPLC separation of Biogel P10 peak IIB material Each fraction (tube No 41, 5 ml) was treated as described in Fig. 2 Black bars, RIA IGF II activity.

Characterization of IGF II peptides

The amino acid composition of peptide IGF IIB was previously found [21] to be identical with that of IGF II.

Analysis by gas-phase sequence degradation revealed peptide IGF IIB (39 residues) to be identical with IGF II. The yield of PTH-alanine in the first cycle was

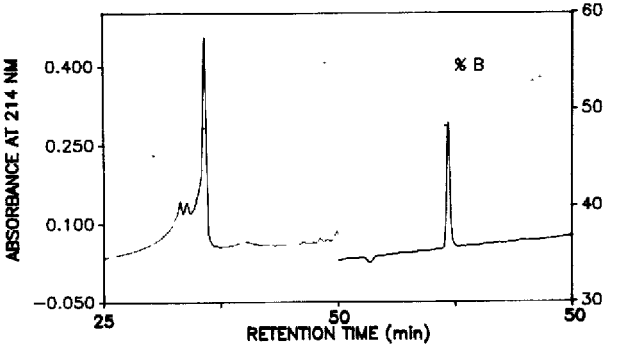

Fig 4 The first immunoactive peak IIB (44 35 min, Fig 3, see Results) was diluted (1 2, v/v) in aqueous 0.08% TFA solution and applied directly to a Vydac C_4 column Mobile phase A was 0 085% (v/v) TFA in aqueous solution (pH 2.1). Mobile phase B was 70% (v/v) acetonitrile in water (final concentration of TFA, 0 08%) Elution was carried out at 0.7 ml/min by a linear increase in the concentration of acetonitrile as follows: 0 min, 30% B, isocratic 0–5 min, 30% B; 5–65 min, 30–60% B, 65–70 min, 60–100% B, 10 min isocratic 100% B, 80–85 min, 100–30% B for a total run time of 120 min Absorbance was monitored at 214 nm between 25 and 50 min Fractions were manually collected Elution profiles· first injection, left panel, second reinjection, right panel

47% and the average repetitive yield was 90.9%. The appearance of the residues Ser-29, Ala-32, Ser-33, Val-35 and Ser-36 excluded the possibility that peptide IGF II B was one of the variant forms reported by Zumstein *et al.* [16] or the variant form described by Jansen *et al* [15] This peptide IGF IIB is identical with the 7.5 kDa IGF II [7]. Moreover, from the sequence data, two variants of peptide IGF IIB seem to exist, one of which (79%) has the amino terminal sequence Ala-Tyr-Arg whereas the other peptide (21%) lacks Ala-1 and begins with Tyr. The significance of this heterogeneity, also found by Rinderknecht and Humbel [7] and by Enberg *et al.* [29] starting from human serum, is not readily explainable.

Structural information on the isolated peptide IGF IIC was obtained by fast atom bombardment mass spectrometry kindly performed by Dr. Becchi (Service Central d'Analyse, CNRS, Vernaison, France). A wide scan gave an average mass of 7811 ± 1 Da for the protonated molecular ion (Fig. 5). Molecular ions corresponding to $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ were detected at 3904 and 2603 mass units, respectively (Fig. 5). The average mass of this peptide IGF IIC is in close agreement with the mass (M = 7811.8) calculated from the predicted amino acid sequence of the IGF II cDNA variant described by Jansen *et al.* [15].

The ability of peptide IGF IIB to stimulate [^{3}H]thymidine uptake into chick

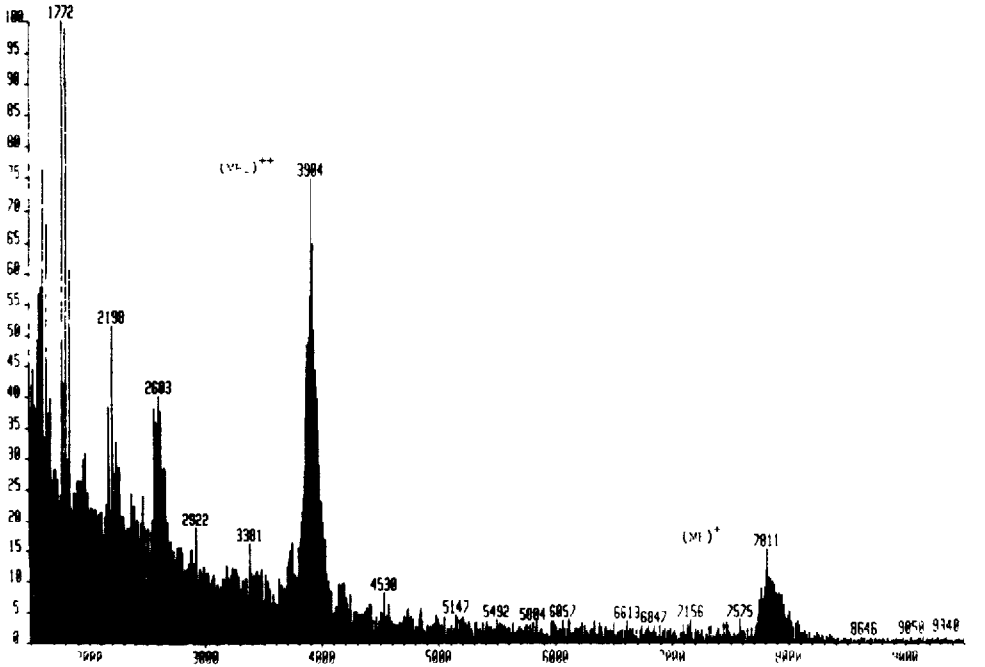

Fig. 5. High-mass region of the fast-atom bombardment spectrum of peptide IGF IIC The spectrum shows the mono-, the di- and the triprotonated molecular ions.

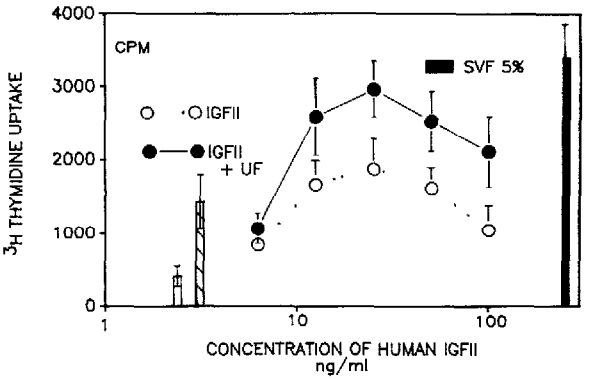

Fig 6 Stimulation of chick embryo fibroblast growth by peptide IGF IIB (○) IGF IIB, (●) IGF IIB incubated with 10% (v/v) of ultrafiltrate (< 1000 Da) of normal human serum, as described in ref. 30. The bars indicate the amount of [^{3}H]thymidine uptake by the medium alone (□), the medium + 10% (v/v) ultrafiltrate (▩) and 5% fetal calf serum (■) Each data point is expressed as the mean of eight values (± S D., bars)

embryo fibroblasts is demonstrated in Fig. 6. The concentration required for half-maximal stimulation was 12 ng/ml. Synergistically with low-molecular-mass growth-promoting factors (LMW-GF) as described and purified by Heulin *et al.* [30], peptide IGF IIB at 20 ng/ml was able to stimulate growth to a level that was 85% of that achieved with 5% of fetal calf serum.

TABLE III

N-TERMINAL SEQUENCE OF PEPTIDE IGF IIB

The amino acid sequence is given in the one-letter notation. ? = PTH amino acid identified but not quantified Sequence cycles that did not yield a detectable amino acid are indicated by a dash. ★ = Complete sequence of IGF II as described by Rinderknecht and Humbel [7].

Polypeptide	Sequence
	1 10 20 30
Peptide IGF IIB	A Y R P S E T L – G G E L V D T L Q F V – G D R G F Y F S R
IGF II*	A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R
	40 50 60
Peptide IGF IIB	P A S R (?) V S R (?) – –
IGF II*	P A S R V S R R S R G I V E E C C F R S C D L A L L E T Y C
	61 67
Peptide IGF IIB	
IGF II*	A T P A K S E

DISCUSSION

This work was carried out to develop a rapid and simple procedure for the preparation of both IGF I and IGF II from human Cohn paste IV, avoiding the use of expensive, large cross-section columns [31] or chromatofocusing steps [24]. This method gives efficient separation and recovery and can be used for laboratory-scale preparation of sufficient amounts of highly purified insulin-like growth factors. The present protocol allowed 107 280-fold purification for peptide IGF IIB, in four chromatographic steps with a recovery of 5–10%.

Advantages of the method are that only one intermediate lyophilization (after desalting on TRIS GF 05) is necessary and that the fractions from Biogel P10 can be directly injected into the HPLC system.

Although TFA has been more widely used as an ionic modifier in protein–peptide separations by HPLC than HCl [32,33], the latter was used in our system for its compatibility with the sulphation bioassay, with an apparently satisfactory resolution. A disadvantage might be the adverse effect of halide ions on stainless steel. After three years, however, we have not observed deleterious effects on our chromatographic apparatus, which is regularly flushed.

Another important advantage is the purification of at least three molecular forms of IGF II as biologically active growth factors: all peptides stimulated DNA synthesis in human and chick embryo fibroblasts [30,34] and are currently in use in binding studies in human lymphocytes and red blood cells [35]. However, complete terminal sequence analysis of both peptide IGF IIA and peptide IGF IIC was necessary to be sure of total homology with the sequence predicted for IGF II variants. The characterization and biological potency of each peptide are currently under investigation.

Our simple method may be adapted for purification on the preparative scale of IGFs from serum of human and non-human species [36] or from culture-conditioned media.

ACKNOWLEDGEMENTS

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