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Application of preparative high-performance liquid chromatography to the purification of a fetal ovine insulin-like growth factor II: N-terminal sequence determinations using two different carriers

J STRACZEK*, M H HEULIN, A M CHENUT, F BELLEVILLE and P NABET

Biochemistry Laboratory, Faculty of Medicine, University of Nancy I, B.P. 184, 54505 Vandoeuvre Cedex (France)

L DENOROY

Central Analysis Laboratory, CNRS, Vernaison (France)

B BARENTON, M BLANCHARD and J CHARRIER

INRA, Zootechnic Station, 34060 Montpellier Cedex (France)

and

J MARTAL

INRA, Physiological Laboratory, 78350 Jouy-en-Josas (France)

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ABSTRACT

A highly efficient procedure for the purification to homogeneity of an ovine fetal insulin-like growth factor II (IGF II) is described Fetal sheep serum was used as the source material, and the bioactivity was followed throughout purification by an IGF II radioreceptor assay Ovine IGF II was isolated by a combination of gel permeation, ion-exchange chromatography and reversed-phase high-performance liquid chromatography The amino-terminal sequence of the first 36 amino acid residues was compared using two supports (polyethylenimine and polybrene) as carrier for protein sequencing Ovine fetal IGF II was found to differ from human IGF II in three residues of the C-domain, with serine, isoleucine and asparagine substituted for alanine, valine and serine, respectively, at positions 32, 35 and 36 The final yield of highly purified ovine fetal IGF II was 134 μg, starting from 450 ml of serum

INTRODUCTION

Insulin-like growth factors (IGFs) or somatomedins are a family of serum polypeptides with insulin- and growth-promoting activity [1,2]. Two forms, IGF I (or somatomedin C [3,4]) and IGF II, have been purified from human plasma [5,6]. Human IGF I is a polypeptide with 70 amino acid residues and is postulated to be the major mediator of growth hormone action on skeletal tissue in postnatal life.

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Human IGF II is also a single polypeptide chain with 67 amino acid residues [7], three disulphide bonds and a molecular mass of 7471 Da. Its physiological role is not clear. Haselbacher and Humbel [8] postulated a possible role in brain development, based on the observation of elevated levels in macro-encephalic patients and a higher proportion of big IGF II form (8–12 kDa) in the human brain and cerebrospinal fluid than in serum. IGF II might also be the major fetal somatomedin: it is present in fetal plasma from rat [9] and sheep [10] at higher concentrations than in the corresponding postnatal plasma. In humans, however, fetal serum IGF II levels have been shown to be lower than corresponding adult values.

Recently, bovine IGF I and IGF II were purified [11] and their primary structures reported to be the same as that of human IGF I or in close homology with human IGF II (64 amino acid residues out of 67 are identical)

The concentration of IGF II-like peptide in the fetal lamb is high and then decreases at term [12], as confirmed by heterologous [13] and homologous radioreceptor assay (RRA). Sheep fetus has proved to be amenable to *in vivo* investigation of the regulation of fetal development. Therefore, the purification and characterization of fetal sheep IGF II are two of our objectives.

This paper describes a method for the isolation of IGF II from ovine fetal serum by preparative reversed-phase high-performance liquid chromatography (HPLC). The final product has been characterized by determination of its amino acid sequence, and it appears to be suitable for tests in preliminary experiments regarding its *in vitro* and as well as *in vivo* use.

EXPERIMENTAL

Ovine fetal serum

Ten pregnant ewes bearing twins were slaughtered at 120 days post artificial insemination. The blood of the 20 fetuses was collected after decapitation and kept on ice. Just after clotting, the blood samples were centrifuged at 3500 *g* for 30 min, and the sera were pooled and frozen at −20°C.

Assays

The biological activity of ovine IGF II was monitored by RRA using plasma membranes from hypophysectomized rat liver and ^{125}I-labelled IGF II, as previously described [14].Liver membranes were used at 150 μg per tube. Synthetic human IGF II (obtained from Lilly Research, Windlesham, U.K.) was labelled with ^{125}I (purchased from Amersham-France) by the Chloramin-T method, and served also as cold ligand to get the standard binding curve After overnight incubation at 4°C in Tris–bovine serum albumin buffer, samples were centrifuged and the pellets counted in a gamma counter (Gammamatic Kontron).

Isolation procedure

To 150 ml of fetal ovine serum, glacial acetic acid was added to a final concentration of 0.5 *M* and the pH was adjusted to 2.8 with hydrochloric acid. The acidified serum was loaded at 275 ml/h onto a column (100 cm × 10 cm I.D.) of Sephadex G-75 (Pharmacia, Uppsala, Sweden), equilibrated in 0.5 *M* acetic acid at room temperature. Fractions of 90 ml were collected automatically over 20 min (Fraction Collector FRAC 300, Pharmacia LKB, Sweden) and assayed for specific IGF II RRA activity. Peak binding fractions were pooled and stirred overnight with 300 ml (packed volume) of SP-Sephadex C-25 (Pharmacia) equilibrated in 2 *M* acetic acid–75 m*M* NaCl (pH 2 84). Active fractions from three chromatographic runs were stirred with the same SP-Sephadex (*i.e.* 450 ml of fetal ovine serum). The gel was washed with 2 l of adsorption buffer (2 *M* acetic acid–0.4 *M* NaCl, pH 2.84) for 30 min, and bound proteins were batch-eluted by washing sequentially with 400 ml of extraction buffer (0.2 *M* NaCl–0.2 *M* ammonium acetate) at pH 5.0 (eluate B, 3 h), then pH 6 (eluate C, 3 h), and then pH 9 (eluate D, overnight), as previously described [15] The contaminating resin particles were carefully removed by two successive 30-min centrifugation runs at 12 000 *g* and 4°C (Cryofuge 20-3, Heraeus). The supernatant was filtered on a glass filter holder (47 mm, 0.45 μm, Millipore, Bedford, MA, U.S.A.), acidified immediately with glacial acetic acid to a final concentration of 1 0 *M* and subjected to preparative reversed-phase chromatography.

Preparative high-performance liquid chromatography

Preparative HPLC was carried out with a gradient liquid chromatograph (Millipore-Waters, Milford, MA, U.S A.), equipped with two Model 510 solvent pumps, a Model 712 WISP autosampler, an M 481 spectrophotometer connected to an SP 4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.) and a Foxy 2200 fraction collector (Isco, Lincoln, NE, U.S.A.).

The chromatographic unit consisted of an Orpegen reversed-phase column (300 Å, 7 μm, 250 mm × 10 mm I.D.; Orpegen, Heidelberg, F R.G.). Separation of polypeptides was performed at room temperature Prior to sample loading, the column was equilibrated overnight with blank gradients (flow-rate 1 5 ml/min): 100% mobile phase 1A buffer (10 m*M* HCl–0 15 *M* NaCl (pH 2.2), as previously described [16]), 0 to 20% mobile phase 1B (70% acetonitrile–30% buffer A) from 0 to 5 min; 20 to 39% 1B from 5 to 23 min; 39 to 47% 1B from 23 to 38 min; 47 to 57% 1B from 38 to 48 min; 57 to 63% 1B from 48 to 58 min; 63 to 100% 1B from 58 to 70 min; 100% 1B for 10 min; 100% 1B to 100% 1A from 80 to 85 min; total run time, 120 min. The sample (20 ml) was loaded via pump A at 2.0 ml/min and, after washing until the absorbance at 280 nm returned to the baseline value, the gradient was started at 2.0 ml/min. Fractions were collected automatically every 0.5 min.

Analytical reversed-phase HPLC

Analytical reversed-phase HPLC was performed at room temperature on a Waters Assoc. automated liquid chromatograph with Maxima 820 data station, configured with an NEC APC IV/Powermate computer and with a photodiode-array detector (Waters 990) connected to an NEC APCIII computer and a Waters 990 plotter. For editing of chromatograms, operating software 990 (revision 3 01) was used.

System 1 The chromatographic unit consisted of a Vydac phenyl column (300 Å, 5 μm, 250 mm × 4.6 mm I D.; Separation Group, Hesperia, CA, U.S.A.) Purification of IGF II RRA active fractions was performed using the following gradient (flow-rate, 0.8 ml/min): 70% mobile phase 2A (0.08% TFA in tri-distilled quartz water)–30% mobile phase 2B [0.08% TFA in acetonitrile–water (70:30, v/v)] from 0 to 5 min; 30–60% linear gradient of 2B from 5 to 65 min; 60–100% 2B from 65 to 70 min; isocratic 100% 2B for 10 min; 100% 2B to 30% 2B in 5 min; total run-time, 120 min. The absorbance was measured at 214 and 280 nm. Fractions were collected manually, sometimes concentrated but never dried in a Speed Vac concentrator, and stored at −70°C in mobile phase until further analysis by HPLC.

System 2. The chromatographic unit consisted of an Aquapore butyl (C_4) HPLC column (300 Å, 7 μm, 220 mm × 4.6 mm I.D.; Applied Biosystems, Santa Clara, CA, U.S A.). Separation of active peptides was achieved with the same gradient as decribed above (flow-rate, 0.6 ml/min). Fractions were collected manually and stored at −70°C in eluent 2B until sequence analysis.

Amino acid sequence analysis

All the products and reagents used for sequencing were from Applied Biosystems (Foster City, CA, U.S.A.), except polyethylenimine, which was purchased from Sigma (St. Louis, MO, U.S.A.).

The sequence determinations were performed by Edman degradation using a 470 A Applied Biosystems gas phase sequencer with an on-line 120 A Applied Biosystems PTH analyzer. An IBM PC-AT microcomputer was connected to the PTH analyzer through a Beckman 406 interface and was used to quantify the phenylthiohydantoin amino acids (PTH-AAs) by means of the System Gold software provided by Beckman

The samples were sequenced using glass fibre coated with polybrene (PB) or polyethylenimine (PEI) as a support. When PB was used, 30 μl of a solution containing 3 mg of PB and 0 2 mg of NaCl were spotted on a TFA-pretreated glass fibre disc. The disc was subsequently precycled in the sequenator using the 03CBGN program. Then the sample was spotted on the disc and sequencing was performed using the 03RPTH program. Both of these programs were provided by Applied Biosystems.

When PEI was used, a TFA-pretreated glass fibre disc was soaked in a 0.3% (w/v) aqueous PEI solution for 2 h, washed with 6 ml of HPLC-grade water

followed by 2 ml of butyl chloride, and dried [20]. The disc was subsequently precycled using the one-cycle 03CBEI program described by Le Caer and Rossier [20]. Then the sample was spotted on the disc and sequencing was performed using the 03CPEI program, which is a modified 03RPTH program [20].

As the exact amount of sample loaded on the sequencer was not known, the initial yield could not be calculated. The repetitive yield was determined through semi-logarithmic regression analysis of all identified PTH-AAs, except PTH-Ser and PTH-Thr, which are recovered in low yield and not accurately quantified.

RESULTS

The isolation of IGF II from ovine fetal serum was achieved using a series of preparative and analytical HPLC steps. The amount of IGF II bioactivity in the individual pools throughout the purification process was determined by a heterologous RRA [14] and by integration of the absorbance peak at 214 nm. Human IGF II (7.5 kDa), purified in our laboratory [16], which has been quantified by total amino acid analysis, was used as standard, and it was assumed that the molar absorption coefficients of these two peptides were the same at 214 nm.

Insulin-like growth factors (IGF I and IGF II) were first separated from IGF-binding proteins by gel permeation on Sephadex G-75 in 0 5 *M* acetic acid [11]. The distribution of eluted protein and IGF II receptor binding activity is presented in Fig. 1. IGF II receptor binding is calculated as the percentage competition for human [^{125}I] IGF II binding to solubilized rat liver membranes. Pooled active fractions (28–36, in Fig. 1) were adsorbed on SP Sephadex C-25 using a modification of the procedure previously described [15,17]. More than 70% of IGF II

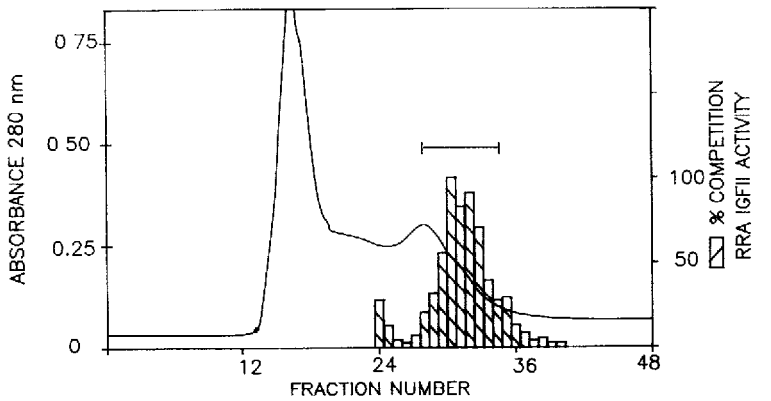

Fig. 1 Elution profile of protein (curve) and RRA binding activity (shaded area) from the G-75 column Acidified fetal serum (150 ml) was applied to a 100 cm × 10 cm I D column equilibrated in 0 5 *M* acetic acid and eluted at 275 ml/h Fractions were collected over 20 min The horizontal bar denotes the fractions 28–36, used for further purification

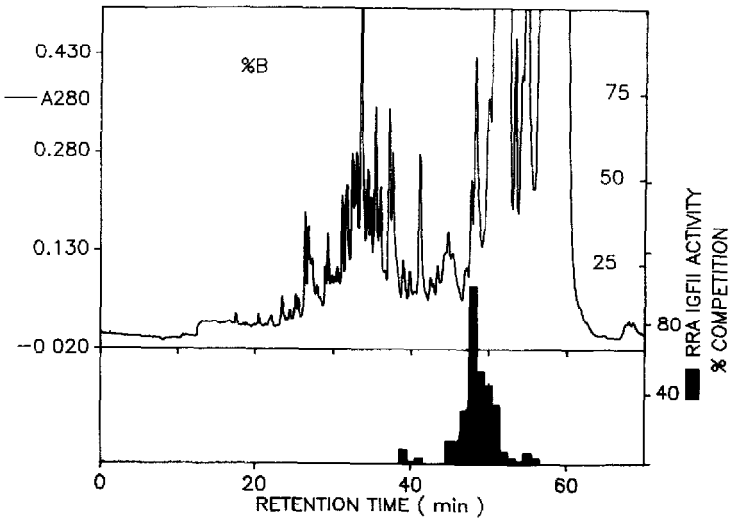

Fig. 2 RP-HPLC separation of eluate D material A 20-ml volume (440 mg of protein), adjusted at pH 2 8, was applied to an Orpegen C_{18} column equilibrated with 10 m*M* HCl 0.15 *M* NaCl (pH 2 1) at 2 ml/min Elution conditions as described in Experimental, *System 1* Absorbance was monitored at 280 nm Fractions were collected every 0 5 min Bioactivity (black area) was estimated as percentage of maximum binding (RRA)

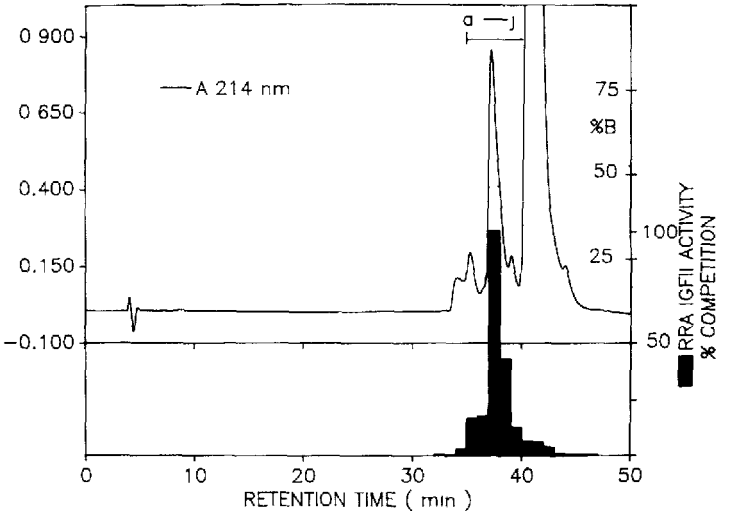

Fig 3 Active fractions from Fig 2 diluted 1 2 with 0 08% TFA and applied directly to a Vydac phenyl column equilibrated as described in Experimental, *System 2* Flow-rate, 0 8 ml/min, the shaded area indicates the active fractions (RRA) The horizontal bar denotes the fractions a–j, collected every 0.5 min (from 35 to 40 min) and used for further purification.

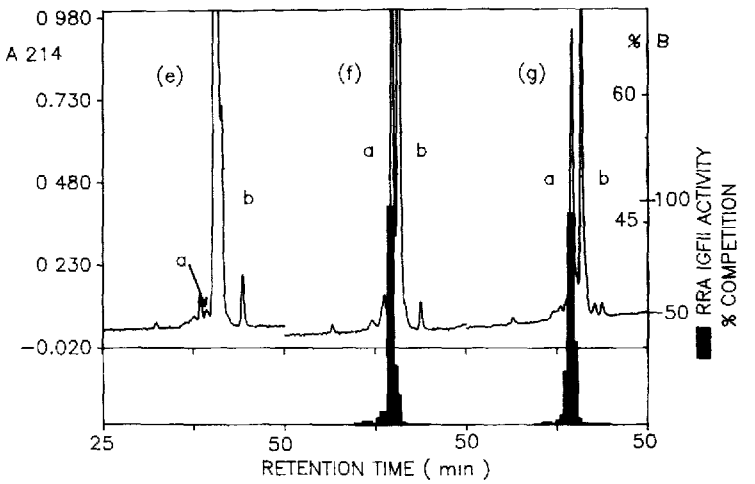

Fig 4 Active fractions from Fig 3 (shaded area) diluted 1 2 in 0.08% TFA and applied to an Aquapore C_4 column as described in Experimental, *System 3* Flow-rate, 0 6 ml/min, the shaded areas indicate the RRA binding activity

receptor binding activity was eluted in 0.2 *M* ammonium acetate–0.2 *M* NaCl (pH 9) (eluate D, *i.e.* 320 ml).

Aliquots of this eluate (20 ml, 22 mg/ml protein, immediately acidified to pH 2.8) were further purified using preparative reversed-phase HPLC, on an Orpegen C_{18} column. A single broad peak of IGF II-like activity was identified (Fig. 2) at 39.9% acetonitrile (48.22 min, 2 7 area percent at 280 nm). Chromatography of this peak on a Vydac phenyl column, on an analytical scale, using TFA as the

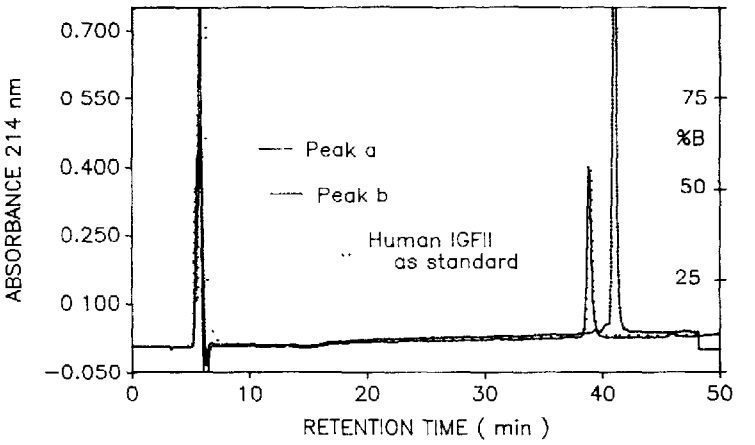

Fig. 5 RP-HPLC of peak a and peak b from fraction f (Fig 4), separately reinjected Conditions as in Fig. 4 Purified human IGF II [16] was used as standard

ion-pairing agent, resulted in two well separated fractions: the first peak (Fig. 3), eluted at 32.3% acetonitrile (37.22 min), contained the majority of the receptor binding activity. Fractions e, f and g (Fig. 3) were re-run separately on an Aquapore butyl column using the above conditions (Fig. 4), and were clearly resolved in two sharp peaks The first peak, labelled peak a, eluted at 33% acetonitrile (39.48 min) and contained most of the competing activity; the second peak, peak b, eluted at 33.7% acetonitrile (40.85 min) By additional re-chromatography on the same column and with the above eluting conditions, these two peaks were purified to apparent homogeneity (Fig. 5), as confirmed by limited amino-terminal sequence analysis.

TABLE I

AMINO ACID SEQUENCE OF THE PEPTIDES CONTAINED IN THE SAMPLE OVIN 3

Ser and Thr, whose PTH are partially destroyed, are not quantified.

Residue number	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Ala	162 5	Asp	165 2
2	Tyr	107 2	Pro	84 4
3	Arg	81 6	Glu	58.4
4	Pro	69 9	Lys	17.6
5	Ser		Thr	
6	Glu	32 7	Val	24 7
7	Thr		Arg	17 1
8	Leu	31 3	–	
9	–		–	
10	Gly	16.7	Thr	
11	Gly	13 9[a]	Ile	7.5
12	Glu	9 3	Ser	
13	Leu	8 2		
14	Val	6 7		
15	Asp	5 2		
16	Thr			
17	Leu	4 7		
18	Gln	4 4		
19	Phe	5 4		
20	Val	2 7		
21	–			
22	Gly	1 5		
23	Asp	1 7		
24	Arg	3 8		
25	Gly	1 1		
26	Phe	1 7		
27	Tyr	1 5		

[a] Not be accurately quantified, because the lags of Gly-10 and Gly-11 are mixed

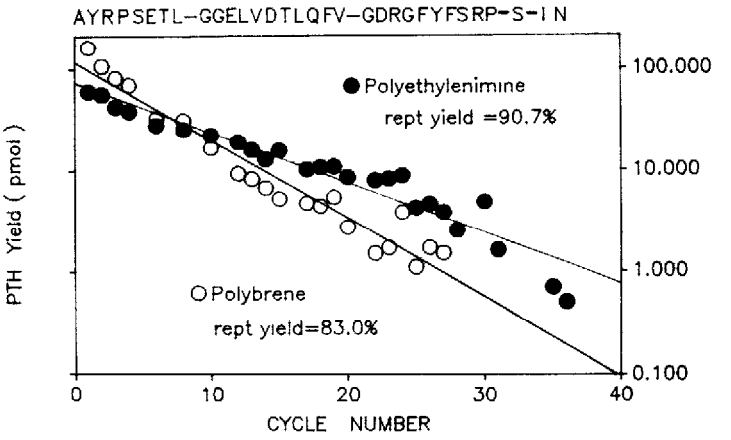

Fig 6 Repetitive yield plots from sequence data of Ovin 3 (○) and Ovin 5 (●) IGF II peptide Amino acids are designated by their one-letter codes. Sequence cycles that did not yield a detectable amino acid are indicated by a dash

In particular, the two fractions were not contaminated by fetal IGF I. Only one PTH-AA was formed at the first cycle of Edman degradation of peak a. The yield of fetal IGF II obtained from peak a was 134 μg from 450 ml of serum, with a total recovery of 23–27% (mean plasma concentration 1300 ± 150 ng/ml [10]).

Amino-terminal sequencing was repeated twice with two different preparations. The sample Ovin 3 was sequenced using PB as a carrier From the first to the twelfth cycle of Edman degradation, two PTH-AAs were formed in nearly equal amounts, except at cycle 8 where only one PTH-AA was identified and at cycle 9 where none was released (Table I). Furthermore, only one PTH-AA was formed from cycle 13 to cycle 27, except at cycle 21 where no PTH-AA was identified. As the sequence of IGF II was recently identified [18], it was possible to assign a sequence for each of the two peptides contained in the sample (Table I) and to calculate the repetitive yields of the Edman degradation, which were 83.0% (Fig. 6) and 76.2% for the longer and the shorter peptides, respectively

The sample Ovin 5 was sequenced to the 36th residue using PEI as a carrier. Only one PTH-AA was formed at each cycle of Edman degradation, except at cycles 9, 21, 32 and 34, where the PTH-AA could not be identified (Table II). The PTH-Ser at positions 29 and 32 were recovered in very small amounts, which were at the limit of detection of the PTH analyzer. The repetitive yield was 90 7% (Fig. 6).

DISCUSSION

In this report, we describe the purification and characterization of ovine IGF II present in the circulation of 120-day-old fetal sheep, an animal that is widely

TABLE II

AMINO ACID SEQUENCE OF THE PEPTIDE CONTAINED IN THE SAMPLE OVIN 5

Ser and Thr, whose PTH are partially destroyed, are not quantified

Residue number	Amino acid	Yield (pmol)
1	Ala	60 6
2	Tyr	56 6
3	Arg	42 7
4	Pro	38 6
5	Ser	
6	Glu	28 2
7	Thr	
8	Leu	25 4
9	–	
10	Gly	22 4
11	Gly	18 1[a]
12	Glu	19 1
13	Leu	16 0
14	Val	12 9
15	Asp	15 8
16	Thr	
17	Leu	10 2
18	Gln	10 7
19	Phe	10 9
20	Val	8 5
21	–	
22	Gly	7 9
23	Asp	8 2
24	Arg	8 8
25	Gly	4 2
26	Phe	4 6
27	Tyr	3 8
28	Phe	2 5
29	Ser	–
30	Arg	4 8
31	Pro	1 6
32	–	
33	Ser	
34	–	
35	Ile	0 7
36	Asn	0 5

[a] Not be accurately quantified, because the lags of Gly-10 and Gly-11 are mixed

used as a model of fetal growth. The use of cation-exchange chromatography coupled with reversed-phase HPLC resulted in a rapid method for preparing highly purified material with an excellent yield. This method may prove useful in the purification of different molecular forms of IGFs from other species or from conditioned media of cultured cells.

Amino-terminal sequencing yielded only a single PTH-AA, indicating that the IGF II isolated in this study is of high purity and is different from its human counterpart where a double sequence is commonly observed: the major sequence (80%) began with Ala-1, and the minor sequence was the des-Ala form [7,16,19].

Two supports, PB- or PEI-coated glass fibre, have been used for the N-terminal sequence analysis of the samples In both cases, the repetitive yields were lower than usual (92%); the origin of this discrepancy is unknown Nevertheless, the repetitive yield was higher with PEI than with PB, and a longer sequence could be determined (36 residues *versus* 27), although a smaller amount of sample was used as shown by the amount of PTH-Ala recovered at cycle 1 (see Tables I and II). The interactions of the Edman degradation by-products are weaker with PEI than with PB. Consequently a smaller volume of solvent can be used for the washing steps, decreasing the occurrence of sample wash-out. In addition, as the by-products are more easily removed, the background on the chromatograms of PTH-AA is decreased, allowing an easier and longer determination of the sequence. Finally, as emphasized by Le Caer and Rossier [20], a better recovery of some PTH-AAs could also contribute to higher repetitive yield when using PEI

No PTH-AA has been identified at positions 9 and 21. It is possible that cysteine residues are at these two positions, as in human IGF II, since PTH-Cys is destroyed during the Edman degradation, giving a "blank" on the chromatograms A sequence determination should be performed on a reduced and alkylated sample in order to identify clearly these two putative cysteine residues.

No PTH-AA has been quantified at position 32, although a PTH-Ser was expected according to the sequence of IFG II. As PTH-Ser is partially degraded and always recovered in low yield, it is possible that such a PTH was released at cycle 32, its amount being under the limit of detection. On the other hand, PTH-Ser has been identified at position 33, as expected from the IGF II sequence. This could be due to a build-up of the lag of PTH-Ser 32 and of PTH-Ser 33, leading to a very weak but identifiable signal at cycle 33.

No PTH-AA was identified at position 34, although PTH-Ile and PTH-Asn were clearly released at cycles 35 and 36, respectively. This suggests that position 34 is occupied by a residue whose PTH is recovered in low yield. Such a residue could be Arg, in agreement with the reported sequence of IGF II.

A somatomedin-like polypeptide has previously been partially purified from adult sheep serum [21], and an IGF II-like polypeptide has also been purified from fetal sheep [18]. Our present report confirmed the sequence data obtained by Francis *et al* [18] and more recently by Mahoney and Adams [22], deduced from the nucleotide sequence of an ovine IGF II c DNA. N-Terminal analysis up to

position 36 revealed three differences from that of human IGF II [7,16]. The change at residues 32, 35 and 36 involved substitution of serine, isoleucine and asparagine for alanine, valine and serine, respectively

Complete characterization and identification of variant forms of ovine IGF II are currently under investigation. The availability of purified peptide assists the investigation of metabolism, fetal growth in particular, in sheep.

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