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Primary sequences of insulin-like growth factors 1 and 2 isolated from porcine plasma

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ABSTRACT

Insulin-like growth factors 1 and 2 were purified from porcine plasma. In addition to the determination of their isoelectric points, the primary structures of both proteins were determined, using low microgram quantities of protein, by the versatile combination of time-of-flight plasma desorption mass spectrometry and automated Edman degradation. Porcine insulin-like growth factor 1 was shown to be homologous to both human and bovine proteins; the type 2 growth factor showed one mutation to both human and bovine type 2 proteins.

INTRODUCTION

The insulin-like growth factors IGF1 and IGF2, formerly called somatomedins [1], have been recognised in recent years to play an important role in the regulation of growth. As growth hormone-dependent peptides excreted by the liver, they exert an endocrine control on the overall growth rate of the organism in postnatal life. Additionally, they are produced in almost all body tissues, where they have paracrine and perhaps autocrine functions in controlling cell proliferation [2].

Strong homology exists among IGF proteins derived from different species [3]. Nevertheless, structural differences occur which may have implications for their biological activities [4,5]. It was therefore of interest to isolate and characterise IGF1 and IGF2 from porcine plasma (PIGF1 and PIGF2, respectively) for comparison of their biological properties and their primary structures with homologous proteins from other sources.

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This paper describes briefly the isolation and purification of PIGF1 and PIGF2 from porcine plasma and, in more detail, the extensive use of time-of-flight plasma desorption mass spectrometry (PD-MS), together with protein microsequencing, for the complete identification of the primary structures of both proteins at the low microgram level. The method is particularly applicable to

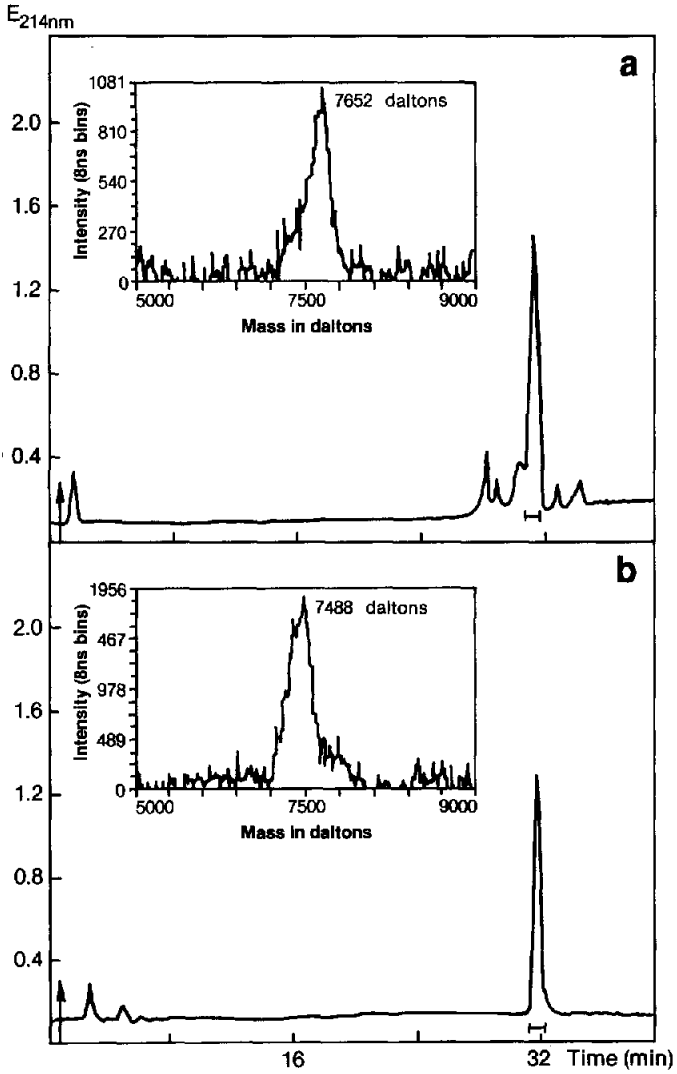


Fig. 1. Final RP-HPLC and PD-MS analysis of PIGF1 and PIGF2. RP-HPLC conditions are as described in Experimental. PIGF1 (a) and PIGF2 (b) were desalted on an analytical Vydac C_{18} columns eluted with a TFA–acetonitrile concave gradient from 15 to 30% over 40 min. Fractions of biological activity were pooled as depicted by the bars in the respective chromatograms. The insets describe mass data obtained by PD-MS for the pooled fractions.

novel proteins, or to recombinant proteins where homologous sequences are already known. During the course of this investigation, Francis *et al.* [5] published the primary sequences of both proteins using a different approach; both sets of results are in complete agreement.

EXPERIMENTAL

Purification of PIGF1 and PIGF2

PIGF1 and PIGF2 were purified as described previously [6,7]. Briefly, plasma was obtained from 7.5-month-old pigs by the addition of citrate to freshly drawn blood. Following centrifugation at room temperature and heat treatment at 83°C, the plasma was filtered at 4°C with Amicon hollow fibres and freeze-dried. The freeze-dried plasma residue was dissolved in 4% formic acid, and IGF-like material was isolated by Sephadex G50 gel permeation chromatography. IGF-rich fractions were pooled and further purified by preparative isoelectric focusing (IEF) (see below). The main components of IGF1-like activity (pH range 8.75–9.05) and of IGF2-like activity (pH range 6.3–6.9) were further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) [6]. The final purification and desalting in a trifluoroacetic acid (TFA)–acetonitrile gradient is shown in Fig. 1a for PIGF1 and in Fig. 1b for PIGF2. Recoveries close to 100% were obtained for the final three HPLC steps. Using this purification procedure, each liter of plasma elicited 0.7 μg of PIGF1 and 14 μg of PIGF2. The overall yields were approximately 1 and 2%, respectively. The preparations were considered to be homogeneous on the basis of the elution patterns and pooling described in Fig. 1, the appearance of a single band in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent autoradiography (data not shown) and time-of-flight PD-MS showing molecules with molecular weights close to those of the human IGF1 and IGF2 (insets of Fig. 1a and b).

Preparation of protein digests

Both proteins (*ca.* 3 μg) were alkylated with 4-vinylpyridine as described previously [8]. The alkylated proteins were then divided into three equal quantities (*ca.* 1 μg). Two portions of each protein were subjected to enzymic digestion as described below; the third portions were analysed by the PicoTag [9] method of amino acid analysis.

Trypsin digestion

Alkylated proteins was dissolved in 40 μl of 0.1 *M* Tris–HCl, pH 8. Digestion was initiated by the addition of 0.1 μg of trypsin and continued for 3 h at 45°C. The total digest was fractionated by RP-HPLC (Figs. 2 and 4 for PIGF1 and PIGF2, respectively) as described below. Fractions were collected manually and each fraction was analysed by PD-MS using a BioIon 20 mass spectrometer (BioIon, Uppsala, Sweden) and, where appropriate, subjected to automated Ed-

man degradation using an ABI 477A protein sequencer (Applied Biosystems, Foster City, CA, U.S.A.) with on-line analysis of the released phenylthiohydantoin (PTH) amino acids.

AspN digestion

AspN digestion was performed as described above for trypsin except that the buffer was 50 m*M* sodium phosphate, pH 7.8, and the incubation was performed at 30°C for 16 h (Fig. 3).

Pepsin digestion

Pepsin digestion was performed as described for trypsin but using 5% formic acid as solvent, 0.01 μg of enzyme and incubating for 2 h at 24°C (Fig. 5).

RP-HPLC

All enzymic digests were fractionated using an ABI 130A chromatograph (Applied Biosystems) over a Brownlee RP300 C_8 (300 Å wide pore, 7 μm particle size, 100 mm × 2.1 mm I.D.) column at a flow-rate of 100 μl/min using a TFA–acetonitrile gradient of 2–80% over 40 min at 45°C. Fractions were detected at 214 nm and collected manually.

Mass spectrometry

The mass spectra were recorded on a BioIon 20. Of each fraction 5 μl were applied to a nitrocellulose-covered target by the spin technique [10]. Spectra were accumulated for 30 min at an acceleration voltage of 18 kV. The mass spectra were analysed by reference to the known sequences of the homologous human proteins by the General Protein Mass Analysis (GPMA) program [11] (tables accompanying Figs. 2–5).

Isoelectric focusing

Flat-bed IEF was performed using an LKB apparatus with a bed of 60 ml Ultradex and a cocktail of ampholines consisting of 0.9 ml pH 5–7, 0.9 ml pH 7–9 and 2.2 ml pH 9–11. The experimental details are described elsewhere [7]. SDS-PAGE was carried out using 15% gels according to Laemmli [12] in a vertical slab apparatus (Bio-Rad).

Radioreceptor assays and immunoassays were performed with pure [^{125}]IGF preparations as described previously [13].

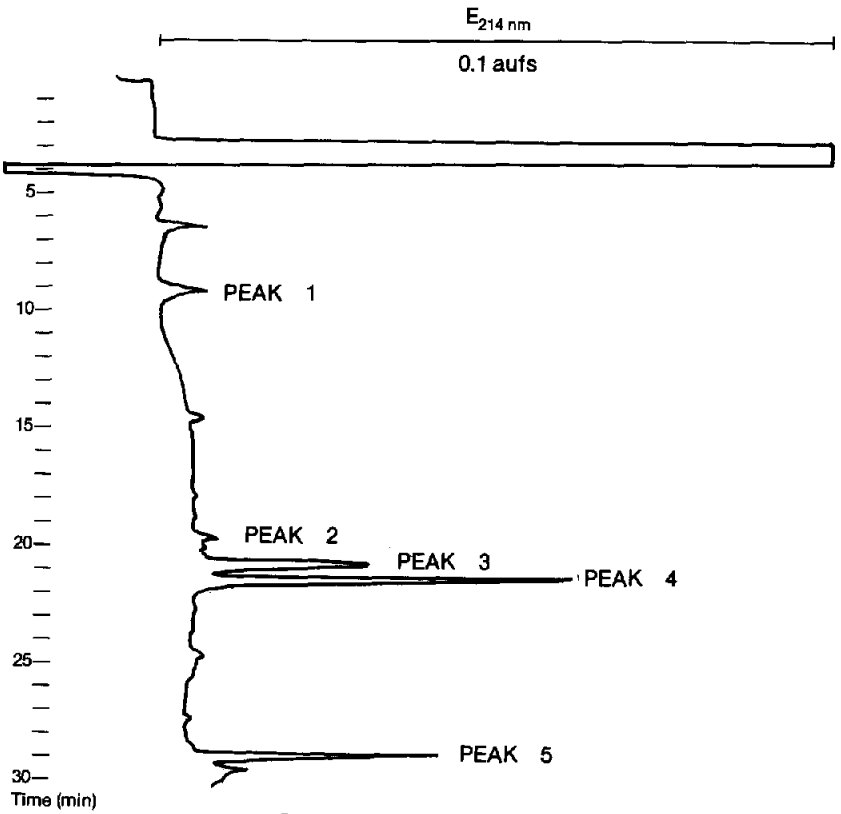
RESULTS AND DISCUSSION

Purification and preliminary biological studies

The overall yields of PIGF1 and PIGF2 obtained from the purification procedure are low, but similar to those obtained for the extractions of the homologous proteins from human plasma or fraction Cohn IV [6,7]. IEF showed that the

isoelectric points of PlGF1 and PIGF2 (8.8 and 6.7, respectively) are essentially identical to those of their human counterparts, using the same analytical conditions [7].

In a preliminary biological study to verify that the isolated proteins were indeed IGFs, an analysis was performed concerning the behaviour of radiolabelled PIGF1 and PIGF2 in two different assay systems and a comparison drawn to radiolabelled human IGF proteins. The results are expressed as the amount (in ng/ml) of unlabelled human IGFs necessary to obtain 50% displacement of the tracer and can be summarised as follows.



Summary of the Tryptic Peptides of PIGF1

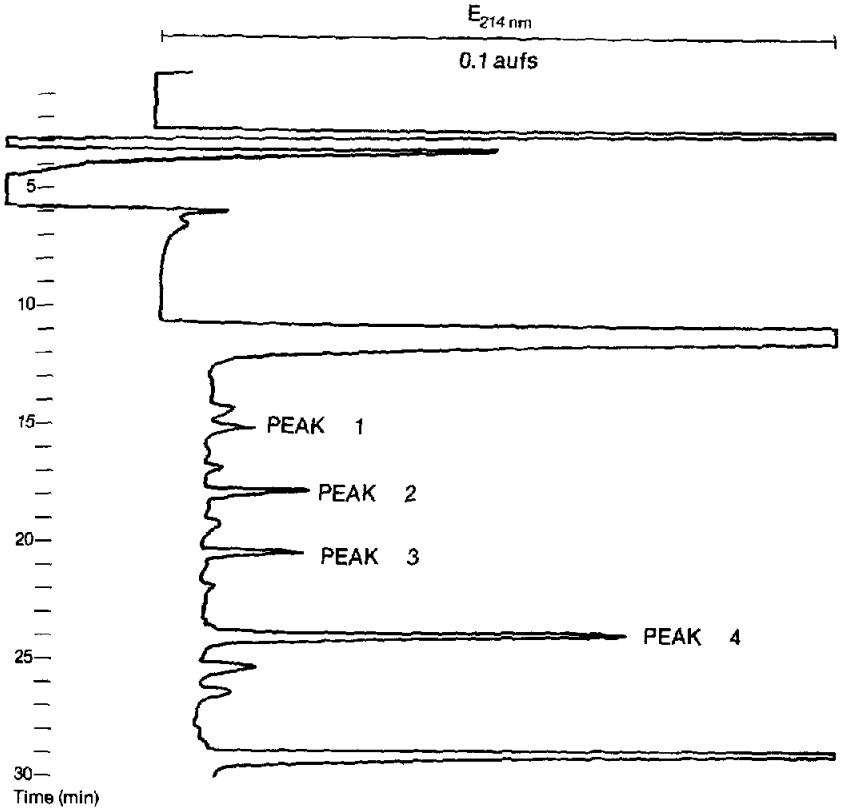
Peak	Mass (daltons)	Peptide Search
1	698.3	51 - 55
2	1806.7	37 - 50
3	1666.6	22 - 36
4	1642.6	56 - 66
5	2405.2	1 - 21

Fig. 2. Trypsin digest of PIGF1. PIGF1 was digested with trypsin as described in Experimental. Collected fractions were analysed by PD-MS and compared to expected peptides from the protein of human origin using the GPMA program. The accompanying table summarises this comparison.

(a) Radioreceptor assay: 0.50 ng/ml was required for ^{125}I-labelled PIGF1 and 0.42 ng/ml was required for ^{125}I-labelled human IGF1; 1.5 ng/ml was required for ^{125}I-labelled PIGF2 and 1.6 ng/ml was required for ^{125}I-labelled human IGF2.

(b) Radioimmunoassay (for IGF1): 0.16 ng/ml was required for ^{125}I-labelled PIGF1 and 0.18 ng/ml was required for ^{125}I-labelled human IGF1.

Identical interaction of both the porcine IGFs with type I and type II IGF



Summary of the AspN Peptides of PIGF1

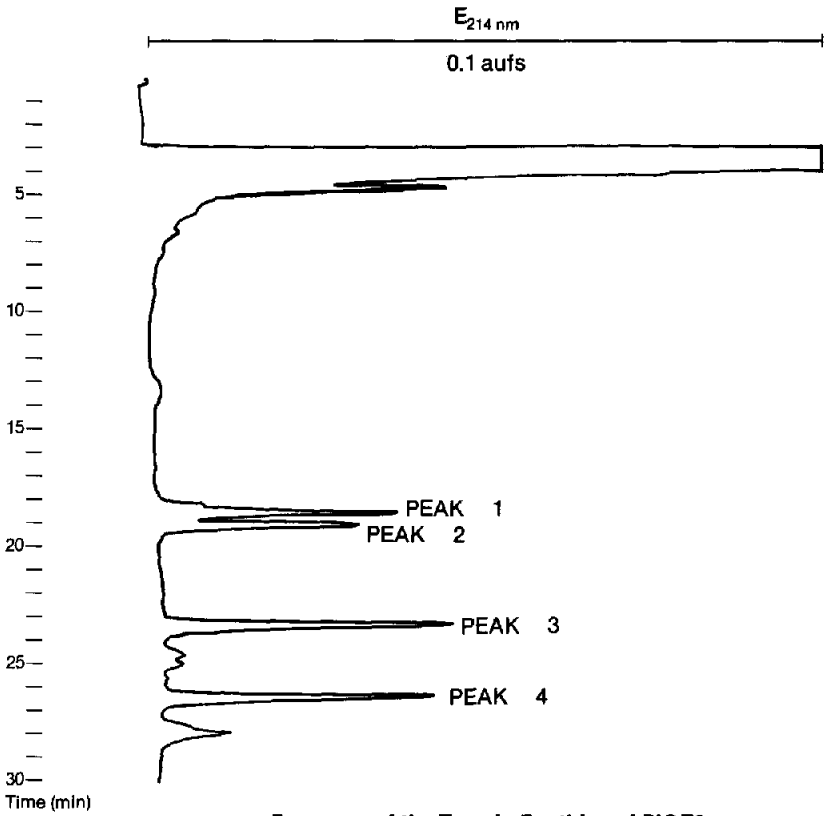
Peak	Mass (daltons)	Peptide Search
1	672.5	53 - 57
2	1276.5	45 - 52
3	1513.7	58 - 70
4	956.7	12 - 19
	1193.0	1 - 11
	2760.7	20 - 44

Fig. 3. AspN digest of PIGF1. PIGF1 was digested with AspN as described in Experimental. Collected fractions were analysed by PD-MS and compared to expected peptides from the protein of human origin using the GPMA program. The large late-eluting peak is undigested protein. The accompanying table summarises this comparison. Peak 4 contains three coeluting peptides.

receptors as well as with antibodies against human IGF1 was shown when compared to the corresponding human proteins.

Primary structure analysis

The general strategy employed for the primary structure determination of the two proteins is to study the fragments obtained from enzymic digests by PD-MS and to relate the molecular weights obtained to those predicted from the human sequence. The tables accompanying Figs. 2–5 indicate that, only in one instance (Fig. 5, PIGF2 pepsin digest) could no correlation to the prediction be found,



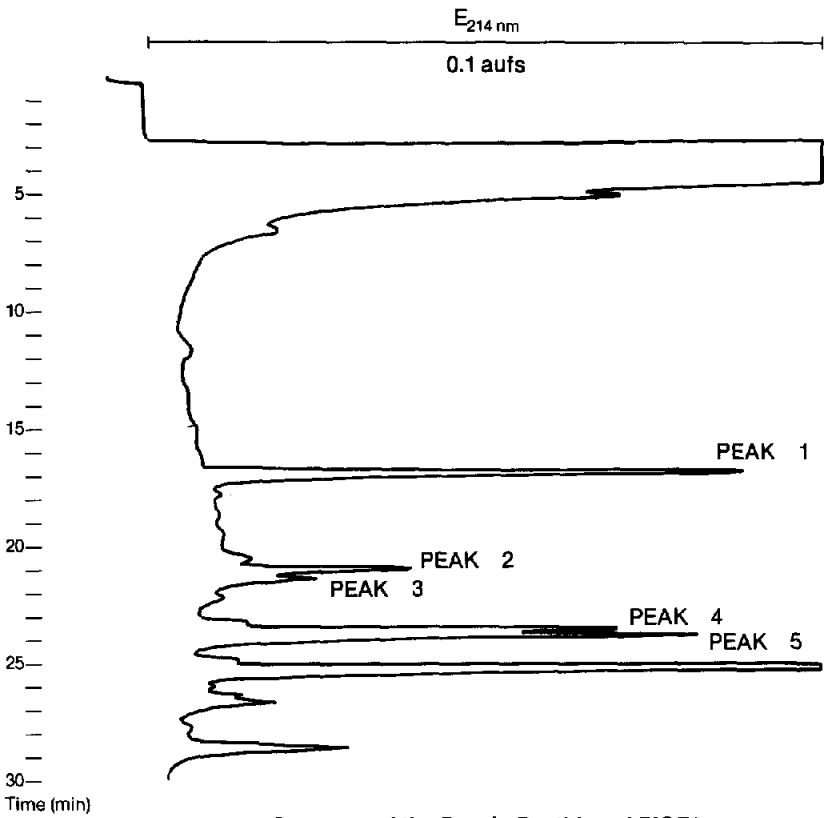
Summary of the Trypsin Peptides of PIGF2

Peak	Mass (daltons)	Peptide Search
1	1266.8	41 - 49
2	1188.4	25 - 34
3	1911.2	50 - 65
4	2840.3	1 - 24

Fig. 4. Trypsin digest of PIGF2. PIGF2 was digested with trypsin as described in Experimental. Collected fractions were analysed by PD-MS and compared to expected peptides from the protein of human origin using the GPMA program. The accompanying table summarises this comparison.

taking into account the relative specificity of pepsin for hydrophobic (leucine and phenylalanine) amino acid residues. The results are in good agreement with those expected since IGF1 proteins from human and bovine origins have the same primary sequences whereas IGF2 proteins from these two sources indicate a variable region between residues 30 and 40; this region is contained in the peptide from the pepsin digest that could not be placed by the PD-MS analysis.

Two enzymic digests were performed on each protein so as to obtain overlapping peptides throughout the primary sequences of each protein. For PIGF1,



Summary of the Pepsin Peptides of PIGF2

Peak	Mass (daltons)	Peptide Search
1	1419.1	56 - 67
2	1168.8	19 - 27
3	1502.1	1 - 13
4	3545.4	?
5	2059.0	1 - 18

Fig. 5. Pepsin digest of PIGF2. PIGF2 was digested with pepsin as described in Experimental. Collected peptides were analysed by PD-MS and compared to expected peptides from the protein of human origin using the GPMA program. The unlabelled late-eluting peak on the chromatogram is partially digested protein. The accompanying table summarises this comparison.

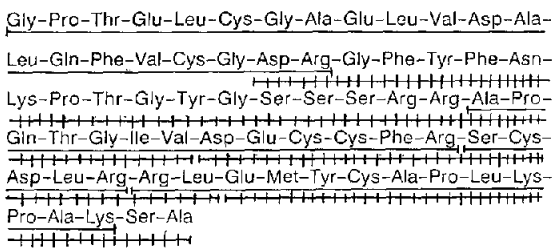


Fig. 6. Overlapping peptides of PIGF1. Summary of the peptides predicted by PD-MS analysis and confirmed by subsequent sequence analysis to constitute the primary structure of PIGF1. ———, Tryptic peptides; + + +, AspN peptides.

trypsin and AspN, a protease which splits on the N-terminal side of aspartate and glutamate residues, were used. As is shown in Fig. 6 overlapping peptides could be obtained throughout the primary structure on the basis of PD-MS analysis alone. The tryptic digest followed by PD-MS analysis of the resulting fractions enabled tentative identification of 68 out of the expected 70 amino acids; a subsequent AspN digest allowed identification of all 70 amino acids. The mass spectrum and subsequent sequence analysis of the AspN peptide containing the expected C-terminal residues is depicted in Fig. 7; the peak at + 16 daltons in the mass spectrum is due to oxidation of the methionine which again is an indication

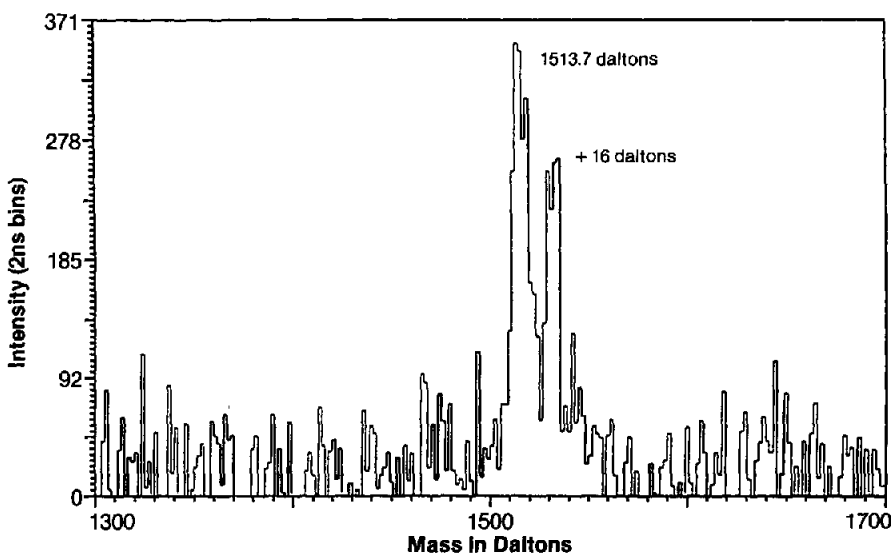


Fig. 7. Mass spectrometric and sequence analysis of peak 3 of the AspN digest of PIGF1. Peak 3 was submitted to PD-MS analysis. Subsequent sequence analysis confirmed it as the C-terminal peptide of PIGF1. Oxidation of methionine can be seen from the mass spectrum but was not evident in the sequence analysis: Glu-Met-Tyr-Pec-Ala-Pro-Leu-Lys-Pro-Ala-Lys-Ser-Ala (Pec = pyridylethylcysteine).

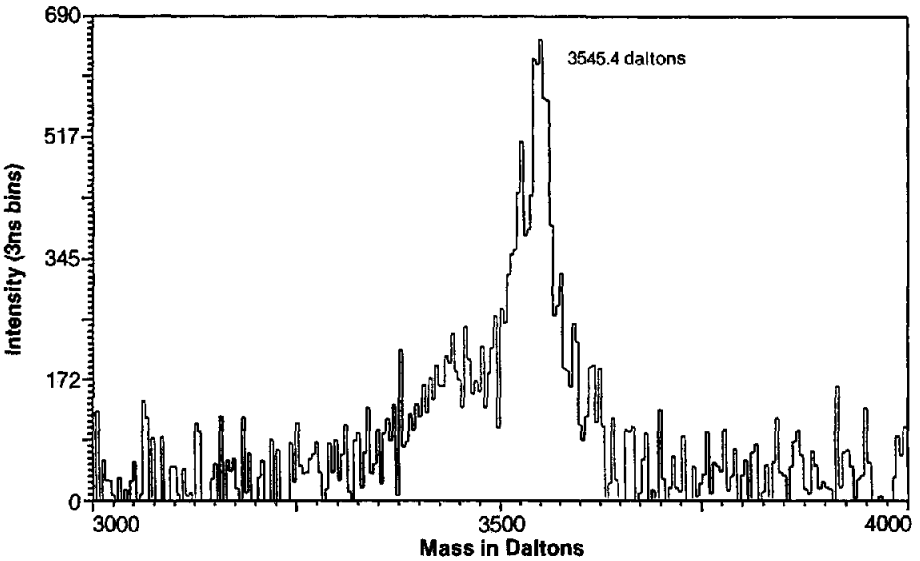


Fig. 8. Mass spectrometric and sequence analysis of peak 4 of the pepsin digest of PIGF2. Peak 4 was subjected to PD-MS analysis. Subsequent sequence analysis indicated an asparagine residue in cycle 9 whereas a serine was expected from the human primary sequence. Sequence analyses: Phe-Ser-Arg-Pro-Ala-Ser-Arg-Val-ASN-Arg-Arg-Ser-Arg-Gly-Ile-Val-Glu-Glu-Pec-Pec-Phe-Arg-Ser-Pec-Asp-Leu-Ala-Leu
(Pec = pyridylethylcysteine).

of which region is being studied. This oxidation probably originates during the mass analysis as it is not evident in the sequence analysis. Sequence analysis was performed on all the overlapping peptides to confirm the suggestions from the PD-MS analysis, hence adding a second dimension to the confirmation of the primary structure of PIGF1.

Trypsin and pepsin were the enzymes of choice for PIGF2. Tryptic digestion prior to PD-MS analysis could tentatively identify 59 out of the expected 67

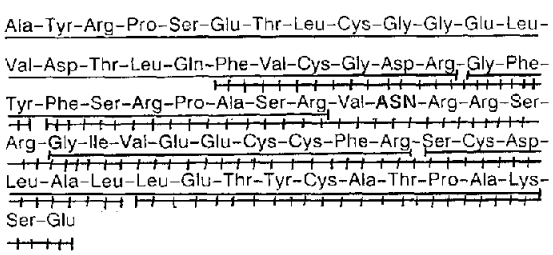


Fig. 9. Overlapping peptides of PIGF2. Summary of the peptides predicted by PD-MS analysis and confirmed by subsequent sequence analysis to constitute the primary structure of PIGF2. ———, Tryptic peptides; + + +, peptic peptides.

TABLE I

AMINO ACID ANALYSIS

Amino acid residue	PIGF1		PIGF2	
	Sequence analysis	Amino acid analysis	Sequence analysis	Amino acid analysis
Ala	6	5.8	4	3.8
Asp	4	4.8	3	3.5
Asn	1		1	
Pec	6	5.6	6	5.7
Glu	4	6.4	6	7.2
Gln	2		1	
Gly	7	7.4	5	5.3
His	0	0	0	0
Ile	1	0.9	1	1.0
Leu	6	6	6	6
Lys	3	2.9	1	1.2
Met	1	0.8	0	0
Phe	4	4.2	4	4.2
Pro	5	5.6	3	3.7
Ser	5	4.6	6	5.6
Thr	3	3.2	4	4.0
Tyr	3	3.1	3	3.0
Val	3	3.0	4	3.9
Trp	0	0	0	0
Arg	6	6.2	8	8.1

amino acids; peptic digestion permitted only identification of 37 amino acids. As mentioned above (Fig. 5), one peptide could not be successfully related to any pepsin peptide of human origin, using the GPMA program. Sequence analysis (Fig. 8) indicated that a serine residue (residue 36 in the human structure) had mutated to an asparagine residue in the protein from porcine plasma. The molecular weight of the corresponding human pepsin peptide is 3516.7 daltons, a difference of 28.7 daltons to the value obtained for the PIGF2 pepsin peptide. The mutation of a serine to an asparagine results in a molecular weight increase of 27 daltons, giving reasonable verification of the sequencing analysis. Fig. 9 presents the overlapping peptides which were confirmed by PD-MS and automated Edman degradation.

Finally, amino acid analyses were performed on the alkylated PIGF1 and PIGF2 proteins. The results, presented in Table I, indicate close agreement of the amino acid composition with that derived by Edman degradation of the individual peptides, indicating that all the amino acid residues had been accounted for. In addition, the molecular weights of the proteins derived from the combined PD-

MS, sequence and amino acid analyses (7648 daltons for PIGF1 and 7495 daltons for PIGF2) are in close agreement with those obtained for the unalkylated, undigested proteins [insets of Fig. 1a for PIGF1 (7652 daltons) and of Fig. 1b for PIGF2 (7488 daltons].

CONCLUSIONS

This paper describes the purification and primary structure characterisation of two IGF-like proteins from porcine plasma, using a combination of PD-MS and automated Edman degradation. The results show that PIGF1 has an identical structure to the homologous proteins from human and bovine origin. PIGF2, on the other hand, shows a single mutation with respect to the corresponding human and bovine molecules, both of which differ from each other by a single mutation. All these mutations occur within a narrow region of the primary structure — around residues 35 and 36. The strategy involved in this study indicates that employing microbore HPLC technology with the sensitivity of PD-MS and peptide microsequencing can permit a rapid and accurate determination of the primary structure of proteins and is particularly amenable to novel proteins whose homologous counterparts in other species already known. The sensitivity of the three techniques involved permits positive identification at the low microgram and low picomole level.

REFERENCES

1 W. H. Daughaday, K. Hall, W. D. Salmon, J. L. van den Brande and J. J. van Wyk, *J. Clin. Endocrinol. Metab.*, 65 (1987) 1075.
2 W. H. Daughaday and P. Rotwein, *Endocr. Rev.*, 10 (1989) 68.
3 J. S Sussenbach, *Progr. Growth Factor Res.*, 1 (1989) 33.
4 I. Zangger, J. Zapf and E. R. Froesch, *Acta Endocrinol.* 114 (1987) 107.
5 G. L. Francis, P. C. Owins, K. A. McNeill, J. C. Wallac and F. J. Ballard, *J. Endocrinol.*, 22 (1989) 681.
6 J. L. van den Brande and C. M. Hoogerbrugge, in A. Pecile and E. E. Muller (Editors), *Growth Hormone and Other Biololgically Active Peptides*, Excerpta Medica, Amsterdam, 1980, p. 81.
7 J. V. L. van den Brande, C. M. Hoogerbrugge, K. Bayreuther, P. Roepstorff, J. Jansen and S. C. van Buul-Offers, *Acta Endocrinol.*, (1990) in press.
8 J. Thomsen and S. Bayne, *J. Protein Chem.*, 7 (1988) 295.
9 B. A. Bidlingmeyer, S. A. Cohen and T. L. Tarvin, *J. Chromatogr.*, 336 (1984) 93.
10 P. F. Nielsen, K. Klarskov, P. Højrup and P. Roepstorff, *Biomed. Environ. Mass Spectrom.*, 17 (1988) 355.
11 P. Højrup, in A. Hedin, B. U. R. Sundqvist and A. Benninghoven (Editors), *IFOS V. Proceedings of the Fifth Symposium on Ion Formation from Organic Solids, Skellefteå, June 1990*, Wiley, Chichester, 1990, p. 61.
12 U. K. Laemmli, *Nature*, 227 (1970) 680.
13 J. Jansen, S. C. van Buul-Offers, C. M. Hoogerbrugge and J. L. van den Brande, *Biochem. J.*, 266 (1990) 513.