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Amino acid modifications in canine, equine and porcine pituitary growth hormones, identified by peptide-mass mapping

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Abstract

Modified amino acid residues in porcine, canine and equine growth hormones purified from pituitary glands were characterised by tryptic mapping and high-performance liquid chromatography with on-line coupled electrospray ionisation mass spectrometry (HPLC–ESI-MS) detection. Hormones from all three species showed the same changes. Conversion of Asp^{128} to iso-Asp^{128} was a component of native hormones, while deamidation of Asn^{12} and Asn^{98} to Asp and iso-Asp, oxidation of Met^{4}, and cyclisation to the pyroglutamyl derivative of Gln^{139}, probably occurred in vitro, during isolation, storage or hydrolysis. Porcine and canine hormones had indistinguishable protein fingerprints, confirming the assumption, based on their cDNA sequences, that their mature primary structures are identical.

Keywords: Peptide mass mapping; Amino acids; Growth hormones

1. Introduction

The anterior pituitary glands of many species, including man, synthesise and secrete growth hormone (GH) in multiple forms [1]. Several post-translational mechanisms contribute to this heterogeneity, including deamidation, acylation, glycosylation, phosphorylation, proteolysis and aggregation. Highly purified pituitary porcine GH (pGH) can be separated into 2–5 subfractions, that differ only in their isoelectric points [2,3]. Several interpretations have been given for this heterogeneity, but the amino acid modifications accounting for the isoform differences are still a puzzle. Violand et al. [4] showed that the pGH residue Asn^{99} can undergo isoaspartate derivatization in alkaline conditions, leading to a shift to a more acidic pI of the modified protein. There is also evidence that this modification may occur in vivo [1].

The aim of the present work was to characterise the GH isoforms found in the pig pituitary gland and to search for possible post-translational modifications in canine and equine GH, whose primary structures are closely homologous to pGH. The cDNA coding sequence of the canine GH (cGH) is identical to pGH, except for a few dissimilarities in the region coding for amino acids which are cut off before secretion [5], and the amino acid sequence of the equine hormone (eGH) differs by only three amino acids. To our knowledge the mature cGH primary

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structure has been only partially characterised [6], and there are no data about the heterogeneity of the cGH and eGH extracted from pituitary glands.

Peptide-mass mapping is one of today's most useful approaches for characterising the primary structure of proteins and peptides [7]. It is based on mass-spectrometry (MS) analysis of the fragments produced after amino acid-specific proteolytic digestion of a protein. The peptide fragment mixture can be analysed and characterised efficiently by coupling the HPLC directly with electro-spray-ionisation MS (ESI-MS). The power of this technique derives from its ability to determine both the retention time and the molecular mass of the eluting compounds (TIC chromatogram). Any changes caused by insertion, deletion or modification of amino acids between proteins will shift the mass and/or retention time of the proteolytic fragments.

The present study describes HPLC–ESI-MS maps based on cleavage on the C-side of lysine or arginine by digestion with the endopeptidase trypsin of pGH, cGH and eGH purified from pituitary glands. The focus was on the identification of modified fragments within the hormone from each species. A pGH produced in bacteria by cDNA technology was analysed to check for pituitary GH purification artifacts.

2. Experimental

2.1. Materials

Porcine GH was purified from pituitary glands as previously described [2]. Canine and equine GH were analytically extracted from 2 g of pituitary (about 10 glands for cGH and 1 gland for eGH) following the procedure used for pGH, and further purified by an HPLC step on a Sephacryl S-200 column (Amersham Pharmacia Biotech, Uppsala, Sweden) in 20 m*M* TRIS–HCl buffer, pH 8.0. The hormones were analysed for purity and homogeneity using SDS-electrophoresis, isoelectric focusing (IEF) and partial NH_2-terminal sequence as previously described [2].

Recombinant pGH (rec-pGH) was obtained from GroPep (Adelaide, Australia). L-Tosylamide-2-phenylethylchloromethylketone (TPCK)-treated bovine pancreas trypsin, sequencing grade, was purchased from Sigma (Sigma–Aldrich, Milan, Italy). HPLC grade acetonitrile was from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA), sequencing grade, from Sigma.

2.2. Trypsin digestion of GH

Porcine, canine and equine GH were dissolved (2 mg/ml) in ammonium bicarbonate buffer, 200 m*M*, pH 8.0. Trypsin was dissolved in the same buffer at a concentration of 1 mg/ml, added to 20 μg of each hormone in a ratio of 1:25, and vortexed. Digestions were run for 1 h and 15 h at 37°C. Reaction was stopped by adding 2 μl of TFA 4N. Samples were frozen and lyophilysed, and stored at −20°C until analysed.

2.3. HPLC–ESI-MS

A quadrupole mass analyser based detector (Micromass Platform LCZ, Micromass, Wythenshawe, UK) was used, fitted with an electrospray ion source. The mass spectrometer was operated in positive ion mode. Intact hormones were diluted to 100 μg/ml with a solution of CH_3CN–H_2O (50:50) containing 0.2% formic acid and introduced into the analyser with a Harvard syringe pump (Harvard Apparatus, Holliston, MA, USA) set at 10 μl/min. The working conditions of the source and mass spectrometer during analysis of the intact proteins were the following: capillary at 2.96 kV, cone voltage ramp from 30 to 90 V, source temperature 100°C, desolvation temperature 150°C, scan range for the mass-to-charge ratio (m/z) from 600 to 2000 u for 4 s with an inter-scan time of 0.15 s, scan mode continuum and photomultiplier at 650 V. Typically, 15 scans were averaged to enhance the signal-to-noise ratio. The instrument was calibrated with a solution of horse heart myoglobin (2 μ*M* in 50% aqueous CH_3CN plus 0.2% formic acid) which covered the m/z range from 600 to 2000. The data were collected and analysed with MassLynx software, and the molecular mass was calculated with Transform, a post-run deconvolution program (Micromass, Wythenshawe, UK).

Trypsin-digested GH were dissolved in 10 μl of 0.1% TFA and 5 μl were injected into a Waters 626

liquid chromatograph. Peptides were separated in a Symmetry300 C_{18} column, 3.5 μm, 2.1×150 mm (Waters, Milford, MA, USA) using an elution gradient of CH_3CN in 0.1% TFA (0–50% in 60 min at a flow rate of 0.3 ml/min). The chromatographic effluent was split 5:1 with 50 μl/min introduced directly into the mass spectrometer interface and the remainder routed to a diode array detector (Waters 996, Waters, Milford, MA, USA) (1 spectrum/s; wavelength from 200 to 320 nm). Mass spectrometric analyses were done in the following working conditions: capillary at 2.96 kV, cone voltage ramp from 30 to 90 V, scan mass range from 125 to 2000 for 2 s with an inter-scan time of 0.1 s, scan mode continuum, source temperature 150°C and desolvation temperature 200°C. Calibration was performed over the m/z range 22 to 2000 with a solution of sodium and caesium iodide (2 μg/μl sodium iodide, 0.05 μg/μl caesium iodide in 50:50 propan-2-ol:water). The data were collected and analysed with the MassLinx software.

3. Results and discussion

The electrospray mass spectra of pituitary porcine, canine and equine GHs and of recombinant pGH are shown in Fig. 1. Each hormone generated about ten multiply-charged molecular ion peaks on the mass-to-charge (m/z) scale. Deconvolution of the multiple charge states yielded a single calculated mass for pituitary pGH and cGH. Equine GH and rec-pGH, showed a second significant component, with masses of 21537.6±0.5 u and 21580.7± 0.8 u, respectively. The decrease of 218 u in eGH and of 281 u in rec-pGH compared to the principal components are consistent with an eGH molecule lacking the Ala-Phe at the C-terminal end and a rec-pGH lacking the Met-Phe residues at the amino-terminal end. Table 1 reports the calculated masses of the three pituitary GH and of rec-pGH, with the theoretical masses calculated from the amino acid sequence. The difference between the masses of rec-pGH and pituitary pGH is due to an additional Met at the N-terminal end of the recombinant protein.

The four hormones were digested with trypsin and the peptides were separated and characterised on an HPLC system interfaced with a mass spectrometer through an electrospray ionisation interface. The sequences of the theoretical fragments based on cleavage of GH on the C-side of arginine and lysine residues and the theoretical and calculated M_r of the peptides produced by digestion of pGH, cGH, eGH and rec-pGH are reported in Table 2. The tryptic peptides were consecutively numbered from the N-terminus to the C-terminus of the primary structures, from T1 to T25. The tryptic fragments from digestion of the four GHs were identified by comparison of the observed and theoretical masses. The majority of eluting fragments were detected as singly charged species, that is $[M+H]^+$ ions. The peptides with mass >2000 were detected as double $[M+H]^{2+}$ and triple ions $[M+H]^{3+}$.

Fig. 2 shows the total ion counting (TIC) chromatograms of tryptic peptide mapping of pGH, cGH, eGH and rec-pGH after 1h digestion. All 25 expected T fragments were detectable in all four species. Fragments T11, a dipeptide, and T19 and T24, that are only of one amino acid, were not detectable as separate fragments, because of interference from background noise and/or elution with the column front (peak at 1.8 min). Nevertheless, they were identified linked to other T peptides. The 1h digestion left the peptide bonds between T2–3, T5–6, T7–8, T11–12, T15–16, T18–19 and T24–25 incompletely digested. Moreover, in the native hormone there are two disulphide bridges, binding Cys^{52} to Cys^{163} and C^{180} to Cys^{188}, accounting for the co-elution as disulphide-linked peptides of T5+T18, T23+T25, T5+T18–19, T5–6+T18–19, and T23+T24–25.

In order to improve the digestion of the combined peptides, the incubation time was therefore prolonged to 15 h. Only the peptide bond between T15 and T16 resisted under these conditions (data not shown). Peaks corresponding to the uncleaved fragments are commonly observed in tryptic maps of GH of many species [8,9].

Between porcine, canine and equine GH sequences there are no amino acid substitutions (Arg or Lys) that can cause alternative sites for trypsin digestion. This explains the similarity between the tryptic digestion chromatograms of the GH of these three species. Nevertheless, only in pituitary pGH and cGH were both the fragment elution pattern and the fragment masses indistinguishable. The peptide

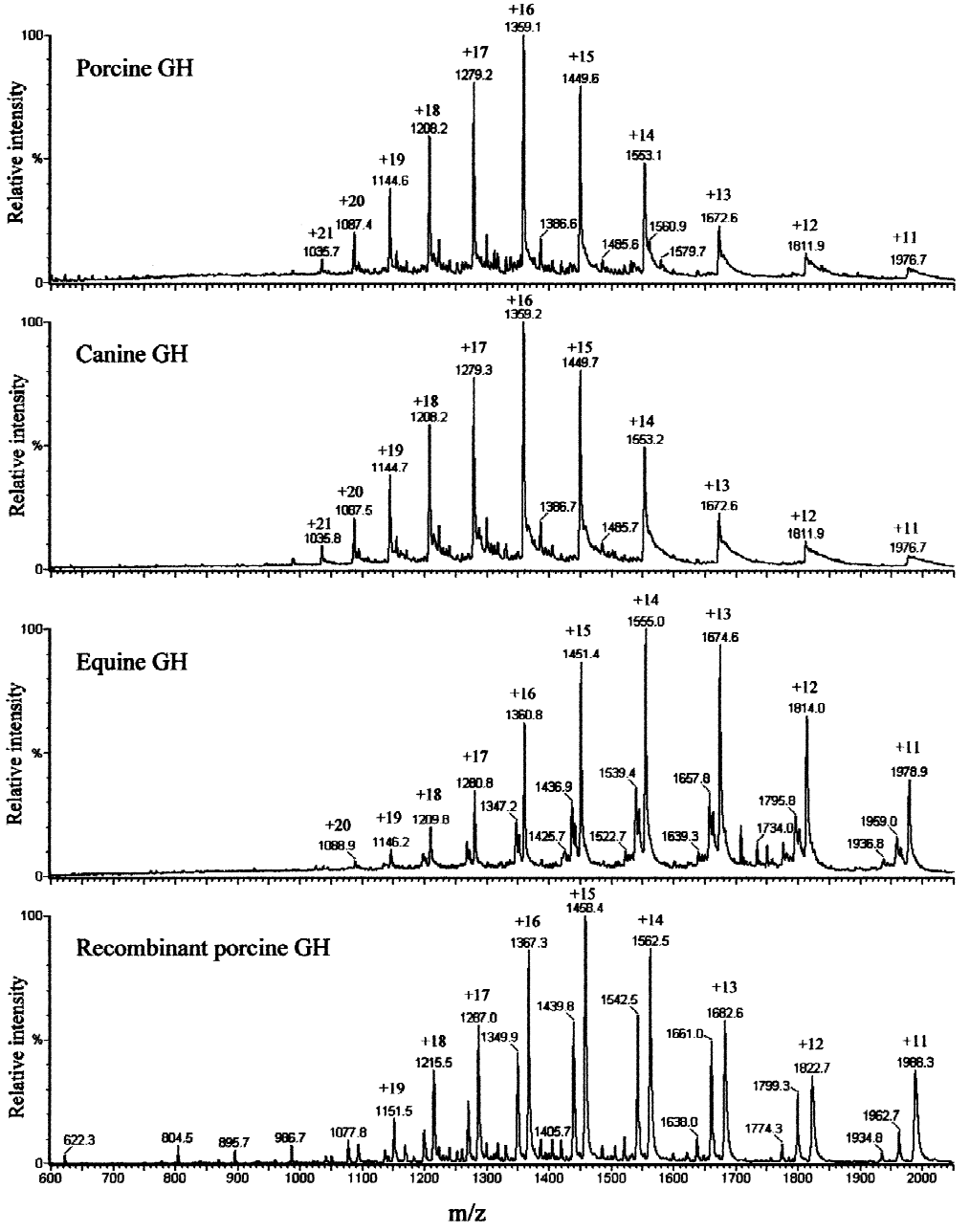

Fig. 1. Electrospray mass spectra of intact pituitary porcine, canine, equine and recombinant porcine growth hormones (GH) obtained by flow injection analysis. The derived charges (z) of the multiply-charged molecular ion peaks on the mass-to-charge (m/z) scale are reported, that are significant for calculation of the molecular mass of the intact GHs.

map of eGH differed from the porcine and canine ones in the position of peptide T7, which eluted slightly later (t_R 26.66 instead of t_R 25.61), very likely as a consequence of the replacement of residue Val[91] with the more hydrophobic Met[91]. Moreover, different masses were measured for peptides T7

Table 1
Theoretical and calculated molecular masses of pituitary porcine, canine and equine growth hormones (GH) and of recombinant porcine GH[a]

GH	Theoretical mass (u)	Calculated (±SD) (u)
Porcine	21730.8	21729.3±1.0
Canine	21730.8	21730.8±0.6
Equine	21756.8	21756.0±0.7
Rec-porcine	21862.0	21861.8±0.5

[a] The electrospray mass spectra were processed to calculate the molecular mass using the Transform software.

(+32 u), T8 (−34 u) and T11 (+28 u), accounting for the three amino acid substitutions within these three fragments in eGH compared to the cGH and pGH sequences. The tryptic mass map for rec-pGH differed from the pituitary hormones in the elution position (t_R 44.49 instead of t_R 43.33) and mass (+131.2 u) of peptide T1, due to the Met^0 at the N-terminal end (numbered as M^0 in order to keep the alignment with the pituitary hormones).

Fig. 3 reports the chromatograms obtained by extracting the masses of fragments T13, T9 and T1 from the 1 and 15 h trypsin digestion TIC chromatograms. The mass corresponding to peptide T13 (902.9 u) was found in two closely eluting peaks (t_R 16.53 and 17.18) in the TIC chromatograms of all three pituitary GHs. This double peak appeared in both the 1 and 15 h fragmentation profiles. Conversion of Asp^{128} to iso-Asp^{128} might account for the peak doubling of this octapeptide. Firstly, this rearrangement of the protein bond occurs through a succinimide intermediate in which the side chain carboxyl of the Asp residue is covalently bound to the back-bone amide nitrogen of the carboxyl-side residue, mainly when this C-flaking amino acid is Ser or Gly in the primary structure [10], as it is for all the three species of GH analysed (Table 2). Second, the rearrangement would not affect the mass of the peptide, while the formation of an atypical isoaspartyl peptide bond could lead to a partial change of the polarity of the peptide, thus affecting the eluting time of the modified fragment. Third, Violand et al. [11] showed in RP-HPLC that a synthetic octapeptide corresponding to pGH T13 where the Asp^{128} was experimentally altered to form iso-Asp^{128} eluted earlier than the normal peptide. The two T13 peaks were seen in peptide mass maps of the three pituitary GH, but not the recombinant pGH, and the iso-Asp^{128} rearrangement was detected even in the milder 1h digestion (differing in that from T9 and T1, as reported below).

Taken together these findings suggest that a GH molecule with an iso-Asp^{128} may be a component of porcine, canine and equine native hormones. However, we cannot exclude that the residue at position 128 in the intact pituitary GH was a succinimide and not iso-Asp, as demonstrated for an experimentally modified rec-pGH [11] and recombinant human GH [12]. Although a succinimide residue would have reduced the GH masses by 18 u (loss of one molecule of H_2O from either Asp or iso-Asp), the concentration of the modified proteins might have been too low to be detectable in the ESI-MS of the not fragmented proteins (Fig. 1). Whether Asp^{128} in pGH, eGH and cGH is transformed as a consequence of the purification or in vivo within the anterior pituitary glands is still not clear. In line with the latter possibility, iso-Asp has been detected as a naturally occurring component in pituitary purified bovine GH [13], and under the conditions used in this paper for isolation of GH from pituitary glands, no Asp to iso-Asp rearrangement should occur.

The mass of peptide T9 (1443.6 u) obtained from all four GHs appeared in a single peak in the 1h fragmentation chromatograms (t_R 30.53 min). However, after 15 h of fragmentation it split into two additional fragment peaks with mass +1 u, which eluted about 1 min earlier and 1 min later than the T9 reference. This 1 u increase can be explained by deamidation of Asn^{98} (NH_2 16 u and OH 17 u), while the subsequent conversion to either Asp^{98} or iso-Asp^{98} could account for the eluting time shifts. Supporting this, Violand's group in another paper [4], demonstrated that isoaspartyl formed at Asn^{98} at neutral and alkaline pH in both pituitary and recombinant pGH, and that the tryptic fragment with iso-Asp substituted for Asn at residue 98 eluted immediately before the normal peptide. We identified the modified T9 peptides in both natural and recombinant GH tryptic mass maps, and they appeared only when the hormones were digested for 15 h. These observations strongly suggest that deamidation of Asn^{98} in eGH, pGH and cGH was probably an in vitro modification arising during hormone digestion.

Table 2
Amino acid sequences of the 25 theoretical fragments (T 1–25) based on cleavage with trypsin of porcine canine and equine growth hormones (GH) and their theoretical and calculated masses

Fragment	Position	Sequence	Theoretical M_r	Calculated Mr			
				pGH	cGH	eGH	rec-pGH
T1	1–16	[M^0]FPAMPLSSLFANAVLR	1735.1 [1866.3]	1734.9	1735.1	1734.7	[1866.0]
T2	17–29	AQHLHQLAADTYK	1496.7	1496.5	1496.7	1496.5	1496.6
T3	30–33	EFER	580.6	580.5	580.5	580.3	580.5
T4	34–41	AYIPEGQR	934.0	933.9	933.9	933.6	934.0
T5	42–63	YSIQNAQAAFCFSETIPAPTGK	2345.6	–	–	–	–
T6	64–69	DEAQQR	746.8	746.7	746.3	746.3	746.9
T7	70–76	SDV^{72}(M^{72})ELLR	831.9 (864.0)	831.8	832.0	(863.8)	831.6
T8	77–94	FSLLLIQSWLGPVQF^{91}(L^{91})LSR	2105.5 (2071.5)	2105.0	2105.2	(2071.4)	2105.6
T9	95–107	VFTNSLVFGTSDR	1443.6	1443.6	1443.7	1443.5	1443.7
T10	108–111	VYEK	538.6	538.3	538.5	538.6	538.5
T11	112–113	LK^{113}(R^{113})	260.4 (288.4)	–	–	(288.5)	–
T12	114–124	DLEEGIQALMR	1275.5	1275.4	1275.3	1275.0	1275.1
T13	125–132	ELEDGSPR	902.9	902.9	902.9	902.5	902.9
T14	133–138	AGQILK	629.8	629.5	629.5	629.7	629.6
T15	139–143	QTYDK	654.7	–	–	–	–
T16	144–149	FDTNLR	765.8	–	–	–	–
T17	150–156	SDDALLK	761.8	761.8	762.0	761.6	761.8
T18	157–165	NYGLLSCFK	1045.2	–	–	–	–
T19	166	K	147.2	–	–	–	–
T20	167–170	DLHK	512.6	512.5	512.5	512.5	512.6
T21	171–176	AETYLR	752.8	752.5	752.5	752.4	752.5
T22	177–179	VMK	377.5	377.5	377.3	377.2	377.3
T23	180–181	CR	278.3	–	–	–	–
T24	182	R	175.2	–	–	–	–
T25	183–190	FVESSCAF	890.0	–	–	–	–
T2–3			2058.3	2058.8	2058.9	2057.9	2058.4
T7–8			2918.5 (2916.5)	2918.0	2918.7	(2916.2)	2918.5
T11–12			1516.8 (1544.8)	1516.5	1516.9	(1544.2)	1516.8
T15–16			1401.5	1401.5	1401.2	1401.1	1401.4
T20–21			1246.4	1246.3	1246.2	1246.3	1246.2
T5+T18			3388.9	3388.7	3388.5	3388.8	3388.5
T5+T18–19			3517.0	3517.0	3516.7	3516.2	3517.0
T5–6+T18			4116.6	4116.5	4116.6	4116.3	4116.4
T5–6+T18–19			4244.8	4244.7	4244.9	4243.7	4245.0
T23+T25			1166.3	1165.1	1165.5	1165.2	1165.0
T23+T24–25			1322.5	1321.3	1321.3	1321.3	1321.3

Amino acid substitutions in equine T respect to porcine and canine T sequences, which are the same, are reported in round brackets. The Met at the N-terminal end of recombinant porcine GH was considered residue No. 0, and is shown in square brackets.

Peptide T1 of all four hormones showed a chromatographic pattern very similar to T9. This fragment (1735.1 u) appeared as a single peak in the chromatogram of the 1h digested GH (t_R 43.00 min), but after 15 h of fragmentation there were two additional peaks with mass +1 u. The residue involved is probably Asn^{12}, that could deamidate to iso-Asp^{12} (eluting 1 min and 30 s earlier) or Asp^{12} (eluting 1 min later). Deamidation of the corresponding Asn in the pituitary bovine GH (bGH Asn^{13}) has been described [14]. However, in the bovine species it is believed to have a physiological role, whereas in pGH, cGH and eGH it might not. In fact, it appeared only after the longer incubations with trypsin, and also in the rec-pGH (although the two deamidated T1 fragments from recombinant pGH gave low signals).

The finding that the deamidation of Asn^{12} and

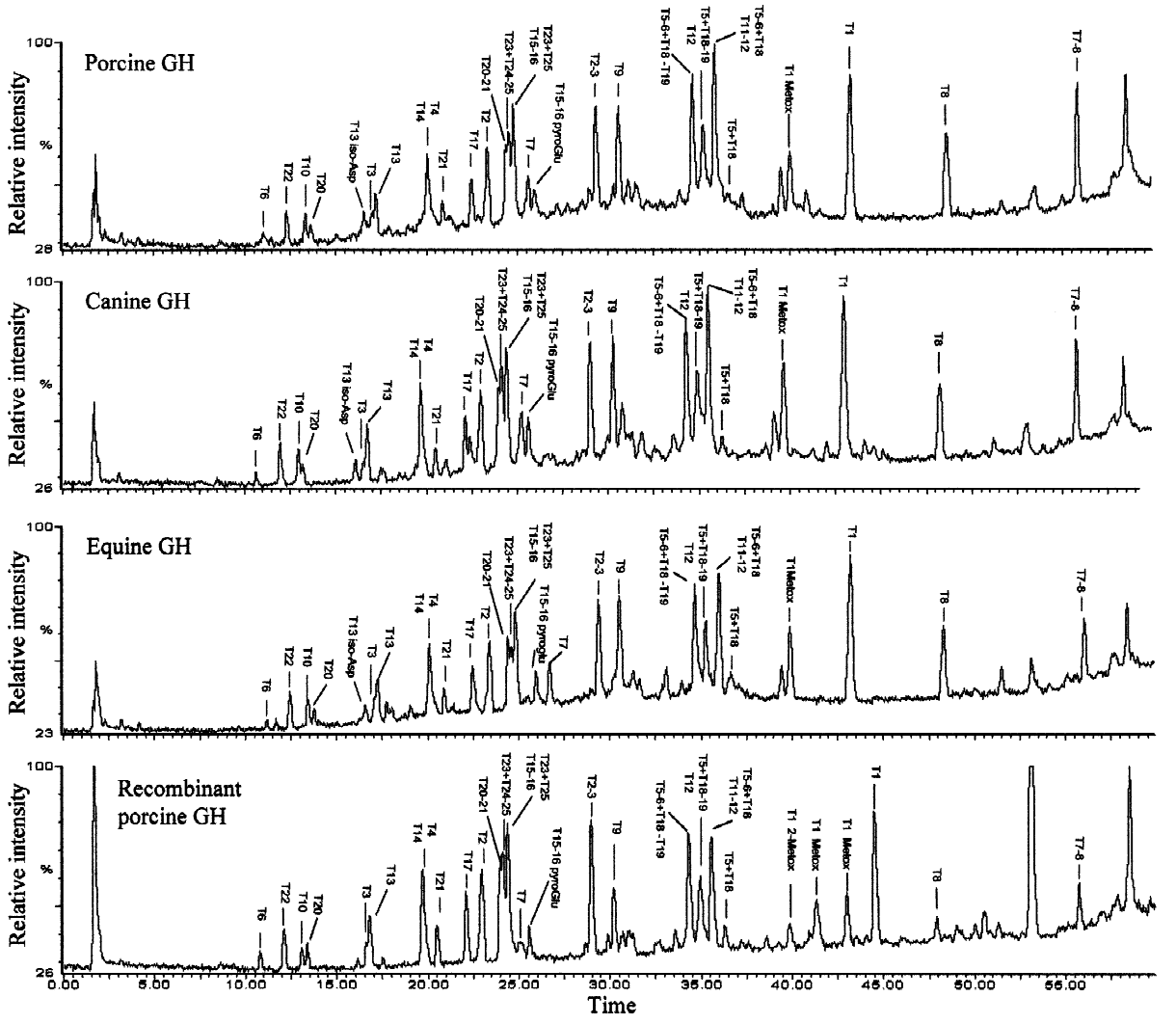

Fig. 2. Total ion counting (TIC) chromatograms of tryptic peptide mapping of pituitary porcine, canine, equine and recombinant porcine growth hormones (GH) from 1h digestions. Scan mass range from 125 to 2000 u. The tryptic peptides eluted in each peak are labelled T, and numbered from the amino terminal. The symbols − and + indicate partially cleaved fragments and disulphide bridges respectively.

Asn^{98} occurs only after tryptic digestion, suggests that the more acidic isoform present in the IEF patterns of pituitary pGH [2], cGH and eGH is not due to these modifications.

The mass of T1 increased by 16 u in another peak in the 1h digestion TIC chromatograms of pituitary GH (t_R 40.00 min instead of the 43.00 min of reference T1), and in two additional peaks (t_R 42.00 min and 43.00 min compared to t_R 44.49) in the rec-pGH (Fig. 1). The recombinant pGH tryptic mass map showed a third fragment peak corresponding to T1 increased by 32 u (t_R 41.30 min). The 16 and 32 u increases are consistent with the oxidation of one and two Met residues to Met-sulfoxide. The fragment peaks were thus identified as being Met^4-sulfoxide T1 for pituitary GHs, and Met^0-sulfoxide, Met^4-sulfoxide and Met^0/Met^4 dioxyl T1 for rec-pGH. The methionine residues near the NH_2 terminus have been reported to be among those most prone to experimental oxidation in human

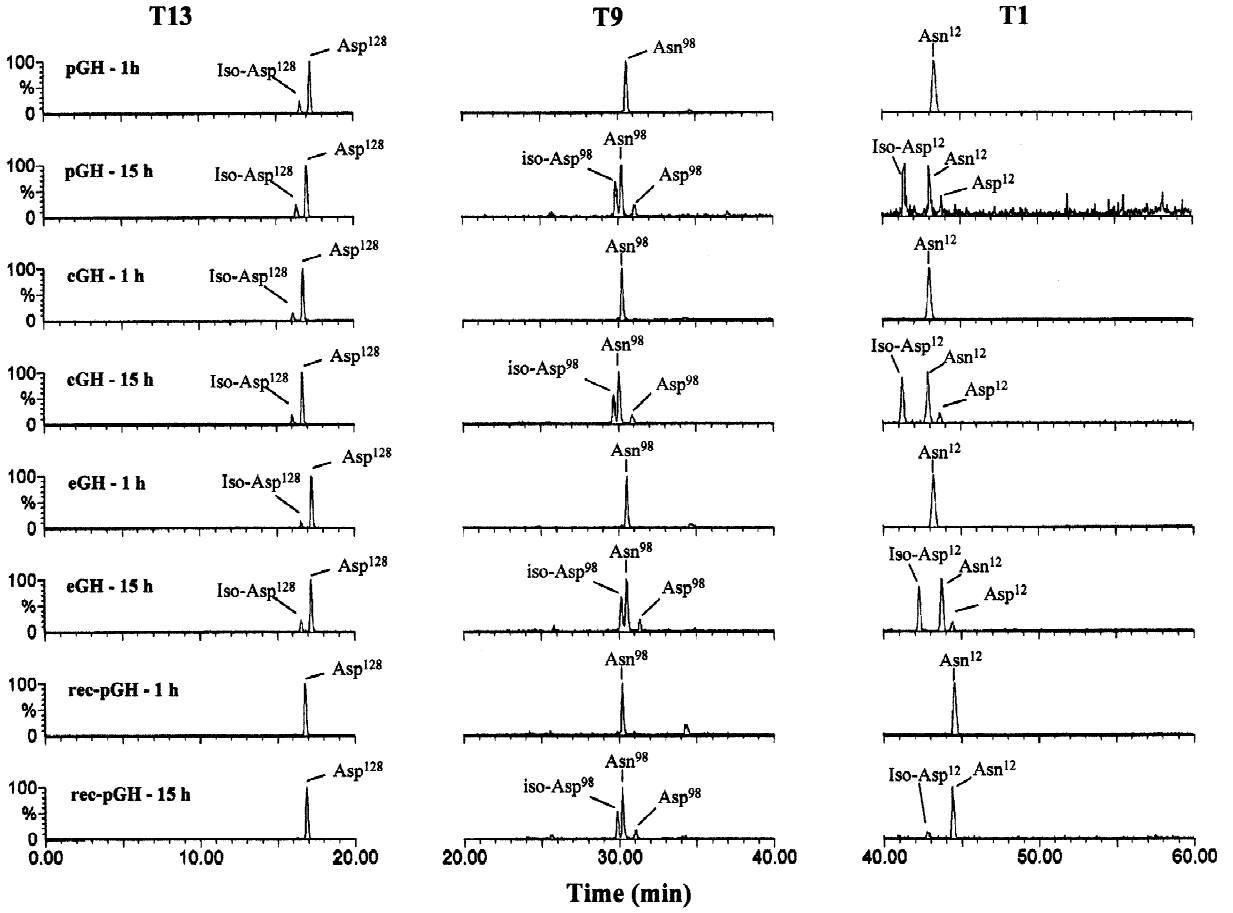

Fig. 3. HPLC–ESI mass chromatograms of tryptic peptides T13, T9, and T1, extracted from the total ion counting (TIC) chromatograms of the pituitary porcine (pGH), canine (cGH), equine (eGH) and recombinant porcine (rec-pGH) growth hormones. For each species the chromatograms from 1 and 15 h digestions are reported, showing the amino acids eluted in each peak. The masses extracted from the TIC chromatograms were: T13=903 u; T9=1444 u; T1=1735 u for pGH, cGH and eGH, and 1866 u for rec-pGH.

GH [15]. This and the identical behaviour of pituitary and recombinant proteins suggest that these modified molecules originated in vitro, during isolation, storage or hydrolysis.

The uncleaved peptide T15–16 was detected in multiple peaks too, in all three pituitary GH and rec-pGH (Fig. 1). The theoretical mass of peptide T15–16 (1401.5 u) was found in the peak eluting at 24.76 min, and with its mass decreased by 17 u in the peak eluting 1 min later. This reduction might be explained by cyclisation to form the pyroglutamyl derivative of Gln^{139}, once exposed as N-terminal amino acid [16].

The combined fragments, including the C-terminus of the four hormones (T23+T25 and T23+T24–25), all showed a mass 1 u lower than the expected values (1166.4 for T23+T25 and 1322.6 for T23+T24–25). The reason for this mass loss is not clear. Nevertheless, both pituitary and recombinant GH showed this modification, indicating that it was not a natural reaction.

In conclusion, the HPLC–ESI-MS tryptic maps obtained in the present study served to locate six sites of amino acidic modification in GH from three different species, with high resolution. An important aspect of the analytical method was the possibility of examining the complex hydrolysates without off-line separation of the tryptic peptides. Among the modi-

fications characterised, only the conversion of Asp^{128} to iso-Asp^{128} seemed to occur naturally. Interestingly, the porcine, canine and equine GH had identical amino acid modifications, despite their different origin. The amino acid sequence homology and the resulting similarity between the three-dimensional structures may create particular microenvironments around definite residues that thus become more prone to change.

Laidler and co-authors [9] successfully distinguished mammalian GHs by peptide mass mapping; they used matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) to analyse recombinant human, equine, porcine and bovine hormones. The peptide-mass maps we report were obtained from GH of pituitary origin, and we added the canine species. To our knowledge this is the first detailed report on the primary structure of the pituitary cGH mature protein. In fact, the amino acid sequence of cGH has only been deduced from the corresponding sequence of the cDNA [5], and the mature protein has only been sequenced for its first NH_2-terminal residue and a few carboxyl-terminal residues [6]. The experimental evidence that canine and porcine GH had an identical protein fingerprint confirms that their protein primary structure is no different.

Acknowledgements

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