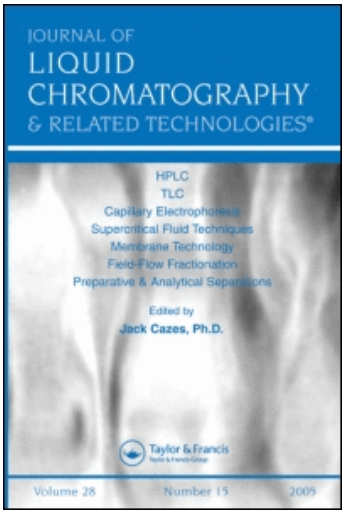

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Separation and Assignment of the Tryptic Peptides of Human Growth Hormone (hGH) and the 20K Dalton hGH Variant by Reversed Phase High Performance Liquid Chromatography

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SEPARATION AND ASSIGNMENT OF THE TRYPTIC PEPTIDES OF HUMAN GROWTH HORMONE (hGH) AND THE 20K DALTON hGH VARIANT BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Reversed-phase high performance liquid chromatography (RP-HPLC) has been used to separate the peptides generated by tryptic cleavage of human growth hormone (hGH) and the 20K dalton human growth hormone variant. The total amino acid compositions of both these pituitary proteins has been accounted for on the basis of these chromatographic mapping procedures. Structural analyses of the peptides isolated from semi-preparative RP-HPLC separations has confirmed that the primary structure of the variant differs from that of the sequence of hGH by deletion of the amino acid residues 32-46.

INTRODUCTION

Two distinct forms of growth hormone occur in extracts of frozen human pituitary glands. In addition to the well characterised 22K dalton version of human growth hormone (22K-hGH), a variant of lower molecular weight can be detected (1, 2) in the extract by polyacrylamide gel electrophoresis in

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sodium dodecylsulphate. This variant, designated 20K-hGH, comprises 5-10% of the growth hormone content of the extract and occurs in various aggregated forms with the majority present as a heterologous dimer with the usual 22K dalton version of hGH. The 20K-hGH variant can be resolved from the 22K-hGH form by ion-exchange chromatography in the presence of 6M urea. Structural studies on the purified 20K dalton hGH by Lewis and co-workers (2, 3) and Chapman et. al. (4) have indicated that the amino sequence of the variant differs from that of the usual 22K dalton form by deletions of amino acid residues 32 to 46. This structural relationship raises the possibility that the deletion is a result of a variation in excision of an intervening gene sequence, a possibility in accord with the observation that an intron has been found (5, 6) in the pre-hGH gene immediately following the DNA sequence coding for residue 31. These findings have stimulated our interest in the mechanism of expression and regulations of the two growth hormone mRNA species as well as in the functional biological differences, including differences in the growth promoting and potentiating diabetogenic activities (7), of these two structurally related proteins. As part of these studies (8), we have utilised reversed-phase high performance liquid chromatography (RP-HPLC) to monitor the isolation and solution properties of these proteins. In this report, we describe the application of these techniques to the separation of the tryptic peptides of hGH and the identification of the aberrant peptides of the 20K dalton variant.

MATERIALS AND METHODS

Equipment

A Waters Assoc. (Milford, Mass.) model 224 liquid chromatograph equipped with gradient elution capability, a U6K sample injector and a model 450 variable wavelength detector were employed. Chromatographic separations were performed on μBondapak C18 columns (30 x 0.4 cm, 10μm) and Radial Pak A cartridges (10 x 0.8 cm). Sample injections were performed with Pressure Lok liquid syringes, series B110 from Precision Sampling (Baton Rouge, La.). The pH measurements were performed with a Radiometer PHM64 pH meter equipped with a combination glass electrode.

Chemicals and Reagents

Orthophosphoric acid and sodium dihydrogen phosphate were ARISTAR grade reagents obtained from BDH (Poole, U.K.), sodium heptanesulphate was obtained from Fluka, A.G. (Switzerland) and ammonium hydrogen carbonate from Ajax Chem. (Aus.). Human growth hormone was isolated from extracts of frozen human pituitaries by the method of Chapman et al. (4) based on the procedure of Lumley-Jones et al. (9). The 20K-hGH variant was obtained from the hGH-dimer pool by fractionation on DEAE-cellulose using a linear gradient of 65mM to 130mM ammonium bicarbonate in 6M

urea. Sigma type XI trypsin (DPCC treated) was used for all the digests. The trypsin was dissolved in 1mM HCl, 2mM Ca Cl_2 at a concentration of 1mg/ml.

AMINO ACID COMPOSITIONAL AND STRUCTURAL ANALYSES

Protein and peptide samples were hydrolysed in vacuo at 110° for 24 hours in 250ul of 6N HCl containing 0.1% phenol. Amino acid compositions were determined on a Jeol Analyser. N-terminal residues were determined by the method of Percy and Buchwald (10) and automated sequential analysis followed established procedures. Reduction and carboxyamidomethylation of the proteins was carried out essentially as described by Cresfield et al. (11). In brief, the hormone (20mg) was dissolved in freshly deionised 6M urea (1.5ml) and 1M Tris-HCl, pH 8.6, (300µl) added. The solution was flushed with nitrogen and β-mercaptoethanol (40µl) was added under a nitrogen barrier and incubated for 2 hours at room temperature. Iodoacetamide (102mg) dissolved in 100mM Tris-HCl, pH 8.6 (700µl) was added to the reaction mixture. Following a 15 min. reaction period, the mixture was chromatographed on a Sephadex G25 column (15 x 0.9cm) equilibrated with 10mM ammonium bicarbonate adjusted to pH 9.5 with ammonium hydroxide. The Cys-(Cm)-protein was recovered by lyophilisation. All enzymatic digests of the proteins and their Cys-(Cm)-derivatives were carried out at 37° using a substrate: trypsin ratio of 100:1. The proteins were dissolved in 50mM Tris-HCl, 2mM $CaCl_2$, pH 7.8, at a concentration of 10mg/ml. Reactions were quenched by the addition of 1/10 volume of a solution of 10mM PMSF/methanol, and the samples lyophilised. The one-letter code for the amino acids is used as described by Dayhoff (12).

CHROMATOGRAPHIC CONDITIONS

All chromatograms were carried out at ambient temperature (ca. 18°). Bulk solvents and mobile phases were filtered through a 0.5um Millipore filter (Millipore Corp., Bedford, Mass.), and degassed by sonication. Flow rates were maintained between 1.0ml/min. and 4.0ml/min. Detection of peptides was at 210nm. All samples were dissolved in the mobile phase corresponding to the initial elution condition with 10-1500µg sample injected in volumes of 10-150µl. The elution fractions were collected manually immediately adjusted to pH7 with 15mM NaOH, the organic solvent partially removed under nitrogen and lyophilised. Analytical and semi-preparative separations were carried out using the standard stainless analytical columns (30 x 0.4 cm). Semi-preparative separations were also carried out using the Waters RCM module with the flexible walled Radial Pak A cartridges (10 x 0.8cm) and 100mM ammonium bicarbonate based eluents. Where peptide assignments or compositional ambiguities arose, recovered peptide fractions were rechromatographed using different elution protocols as described previously (13-15).

RESULTS AND DISCUSSION

The use of RP-HPLC for the structural mapping of polypeptides and proteins is now a well established technique (for recent reviews, see ref. 15, 16). Compared to conventional methods for analytical or micro-preparative separation of protein digests, RP-HPLC procedures offer numerous advantages including short analysis times, generally good sample recoveries and ready appraisal of the homogeneity of the eluted components. Because of the excellent reproducibility and resolution which can be achieved with these RP-HPLC procedures, they also permit direct assessment of the enzymatic digestion conditions including the optimal time course. The potential of these methods to map homologous proteins has been exploited previously in studies reported (17-20) from this laboratory including the identification of hemoglobin variants and phosphorylated forms of rat caseins. In view of the close structural relationship between hGH and the 20K dalton variant, we anticipated similar methods would simplify the separation and analysis of the peptides derived from the tryptic digestion of these two proteins.

In preliminary experiments, the progress of the tryptic digestion of hGH as a function of time was followed by analytical separations (10-150μg protein digest) on a μBondapak C_{18} column using a linear 60 min. gradient from 0.1% orthophosphoric acid to 50% acetonitrile - 50% water - 0.1% orthophosphoric acid (17) at a flow rate of 2ml/min. Representative chromatograms covering digestion times from 15 min. up to 6 hours are shown in Fig. 1. Under these chromatographic conditions 22K-hGH elutes with a retention time of 55.6 min. As is evident from Fig. 1, no significant changes in the elution profile of the native 22K-hGH were obtained with digestion periods longer than ca. 60 min. However, to ensure complete digestion of the 22K-hGH and the 20K dalton variant, a digestion time of 6 hours was employed in the comparative studies.

Comparison of the elution profiles (Fig. 2) obtained for the separation of the tryptic peptides of 22K-hGH (1500μg) and the 20K dalton variant (1200μg) clearly shows the close similarity between these two proteins. Compositional analyses of the various major peaks are provided in Tables I and II. The elution profile for the separation of the tryptic digest of hGH (1400μg) on the Radial Pak A column using a linear 3 hours gradient from aqueous 100mM ammonium bicarbonate to 50% acetonitrile - 50% water - 100mM ammonium bicarbonate is shown in Fig. 3. For clarity of presentation in Table II, the tryptic peptides of the 20K dalton variant are numbered accordingly to the order they would appear in the final sequence of the protein rather than by alignment with hGH itself. Inspection of the composition and chromatographic data reveals several salient features. Firstly, the sum of the amino acid compositions of the tryptic peptides 22K-hGH (T-1 to T-21) shown in Table I and of the tryptic peptides of the 20K dalton

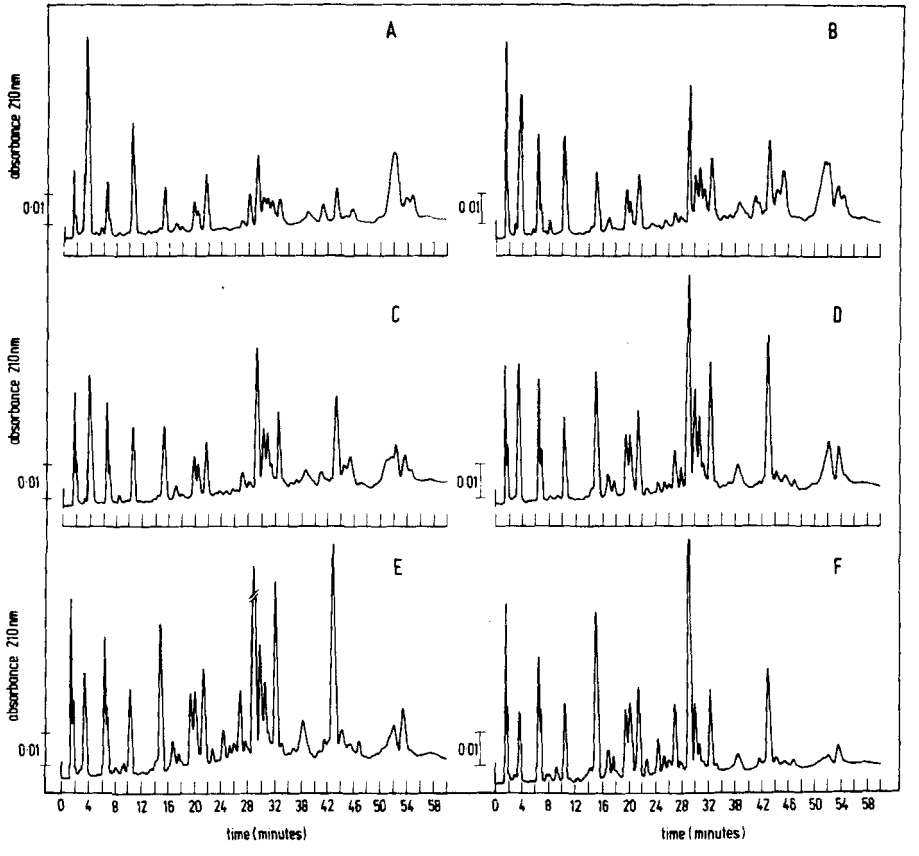

Figure 1. Time course of tryptic (DPCC-treated) digestion of the 22K dalton human growth hormone. All digestions were carried out in 50mM Tris-HCl, 2mM Ca Cl_2, pH 7.8 buffer at 37° using a protein: enzyme ratio of 100:1. Reactions were quenched at 15, 30, 60, 120, 240 and 360 min. by the addition of 10mM PMSF methanol and samples (ca. 150µg) lyophilised. Chromatographic conditions: column, µBondapak C_{18}; flow rate, 2ml/min.; mobile phase, 60 min. linear gradient from 0.1% orthophosphoric acid to 50% acetonitrile - 50% water - 0.1% orthophosphoric acid.

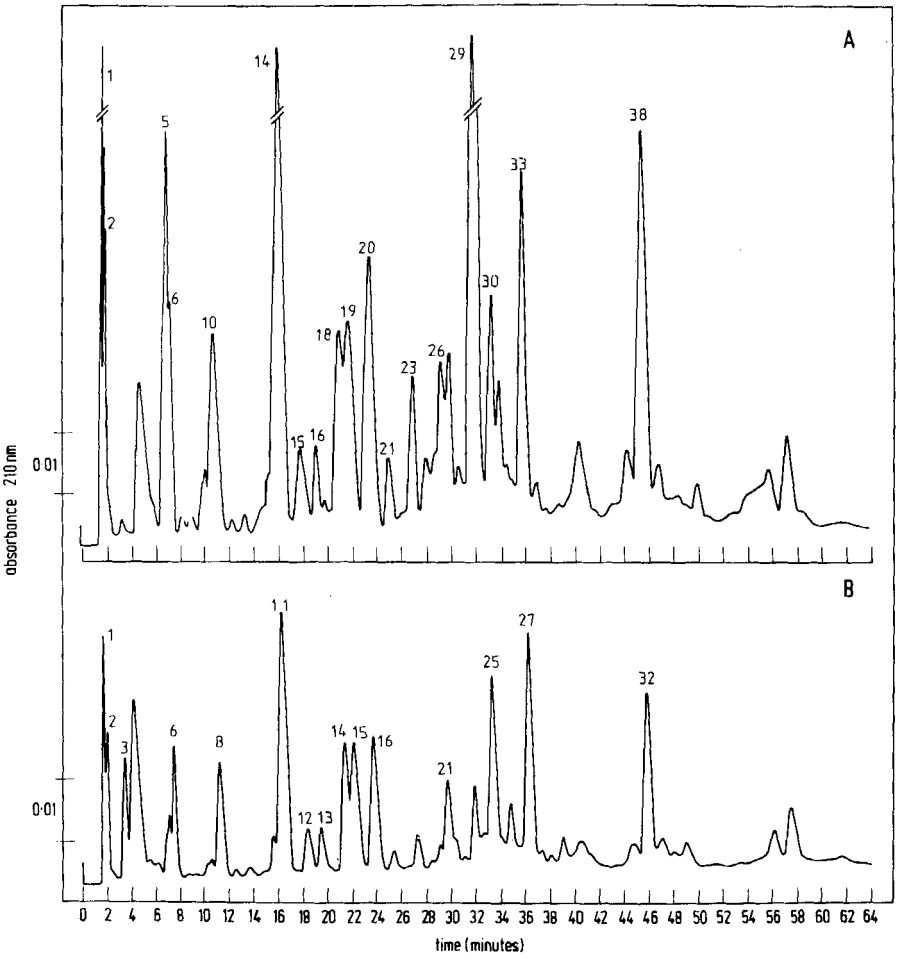

Figure 2. Semi-preparative reversed phase separations of (A) 22K-hGH digest (1500μg) and (B) 20K-hGH digest (1200μg). Chromatographic conditions as in Figure 1.

TABLE I

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES* OF INTACT hGH SEPARATED BY RP-HPLC

Amino Acid	T-1	T-2	T-3	T-4	T-5	T-6 + T-16$^{\nabla}$	T-7	T-8	T-9	T-10	T-11
	1-8	9-16	17-19	20-38	39-41	42-64 + 159-167	65-70	71-77	78-94	95-115	116-127
Asx		2.0(2)		1.1(1)		3.0(3)		1.1(1)		3.8(4)	1.1(1)
Thr	0.9(1)			0.7(1)		2.3(2)	0.8(1)				1.2(1)
Ser	1.1(1)					4.6(5)		0.7(1)	1.6(2)	3.7(4)	
Glx				4.2(5)	2.1(2)	3.4(3)	3.6(4)	1.0(1)	2.8(3)		2.8(3)
Pro	1.8(2)			1.1(1)		2.7(3)			0.8(1)		
Gly						1.0(1)				0.9(1)	1.7(2)
Ala		1.0(1)	1.0(1)	1.8(2)						2.0(2)	
Cys						1.4(2)					
Val									1.1(1)	2.5(3)	
Met		0.9(1)									0.8(1)
Ile	0.9(1)			0.9(1)		1.1(1)			2.0(2)		0.8(1)
Leu	1.1(1)	2.0(2)		2.0(2)		4.1(4)		2.9(3)	4.8(5)	3.1(3)	2.0(2)
Tyr				1.8(2)		2.8(3)				1.8(2)	
Phe	0.8(1)	1.0(1)		1.8(2)		2.8(3)			1.2(1)	1.0(1)	
His			0.9(1)	1.0(1)							
Lys				0.9(1)	1.0(1)		1.1(1)			0.9(1)	
Arg	1.0(1)	1.0(1)	1.1(1)			1.8(2)		1.0(1)	1.0(1)		1.0(1)
Trp									a(1)		
Total Residues	8	16	19	38	41	73	79	86	103	124	136
Peak	20	18	1	29	1	33	2	15	38	30	26

* Peptides are numbered in order of their position in the final sequence.
\+ Amino acid sequence numbers.
$^{\nabla}$ Not present in the Cys(Cm)-hGH digest, but replaced by peptide T-6 (YSFLQNPQTSLCFSESIPTPSNR) and peptide T-16 (NYGLLYCFR).
The values in parentheses are the number of residues based on the sequence.

TABLE I (Continued)

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES* OF INTACT hGH SEPARATED BY RP-HPLC

Amino Acid	T-12	T-13	T-14	T-15	T-17	T-18	T-19	T-20 + T-21[K]	Total
	128-134	135-140	141-145	146-158	168	168-178	169-178	179-183 + 184-191	
Asx	1.1(1)			5.1(5)		1.6(2)	1.8(2)		20
Thr		0.6(1)	0.9(1)	1.1(1)		0.8(1)	0.8(1)		10
Ser	1.0(1)		1.0(1)	0.9(1)				1.9(2)	18
Glx	0.9(1)	1.1(1)	0.7(1)			1.2(1)	1.1(1)	2.1(2)	27
Pro	0.8(1)								8
Gly	1.0(1)	1.0(1)						1.8(2)	8
Ala				1.1(1)					7
Cys								0.5(2)	4
Val						0.9(1)	0.8(1)	1.7(2)	7
Met						0.6(1)	0.9(1)		3
Ile		0.9(1)						0.8(1)	8
Leu	1.1(1)			1.9(2)		1.1(1)	0.8(1)		26
Tyr			0.9(1)						8
Phe		1.0(1)		1.0(1)		0.9(1)	0.9(1)	1.0(1)	13
His				0.9(1)					3
Lys		0.9(1)	1.0(1)	1.0(1)	1.0(1)	2.0(2)	0.7(1)		9
Arg	1.1(1)					1.1(1)	1.0(1)	1.0(1)	11
Trp									1
Total Residues	143	149	154	167	168	178	178	191	191
Peak	6	10	5	14	1	16	19	14	

* Peptides are numbered in order of their position in the final sequence.
+ Amino acid sequence numbers.
∇ Not present in the Cys(Cm)-hGH digest, but replaced by peptide T-6 (YSFLQNPQTSLCFSESIPTPSNR) and peptide T-16 (NYGLLYCFR).
κ Not present in the Cys(Cm)-hGH digest, but replaced by peptide T-20 (IVQCR) and peptide T-21 (SVEGSCGF).
a Not quantitated.
The values in parentheses are the number of residues based on the sequence.

TABLE II

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES* OF 20K DALTON hGH SEPARATED BY RP-HPLC

Amino Acid	T-1	T-2	T-3	T-4	•	T-14√	T-5	T-6	T-7	T-8	T-9	T-10	T-11
	1-8	9-16	17-19	20-49	+	144-152	50-55	56-62	63-79	80-100	101-112	113-119	120-125
Asx		2.0(2)		4.3(4)				1.1(1)		4.0(4)	0.8(1)	1.1(1)	
Thr	1.0(1)			2.6(3)			1.0(1)				1.0(1)		0.9(1)
Ser	1.0(1)			3.1(4)				0.8(1)	1.5(2)	2.8(4)		0.9(1)	
Glx				4.9(5)			4.0(4)	1.1(1)	3.0(3)		3.0(3)	1.1(1)	1.1(1)
Pro	2.0(2)			2.9(3)					0.8(1)			0.8(1)	
Gly				1.1(1)						1.2(1)	2.0(2)	1.0(1)	1.0(1)
Ala		1.0(1)	0.9(1)	1.1(1)						2.0(2)			
Cys				0.9(2)									
Val									1.1(1)	2.3(3)			
Met		0.9(1)									0.7(1)		
Ile	0.9(1)			1.2(1)					1.9(2)		0.9(1)		1.0(1)
Leu	1.2(1)	1.9(2)		4.8(5)				2.6(3)	4.7(5)	3.1(3)	2.1(2)	1.0(1)	
Tyr				2.6(3)						1.5(2)			
Phe	0.9(1)	1.2(1)		3.6(4)					1.2(1)	1.0(1)			1.0(1)
His			0.8(1)	0.8(1)									
Lys							1.0(1)			0.8(1)			0.9(1)
Arg	0.9(1)	0.9(1)	1.0(1)	1.9(2)				0.9(1)	1.0(1)		1.0(1)	1.0(1)	
Trp									a(1)				
Total Residues	8	16	19	68			74	81	98	119	131	138	144
Peak	16	14	1	27			2	12	32	25	21	6	8

• Peptides are numbered in order of their position in the final sequence.
+ Amino acid sequence numbers
√ Not present in the Cys-(Cm)-20K-hGH digest, but replaced by peptide T4 (LHQLAFDTYQEFNPQTSLCFSESIPTPSNR) and peptide T-14 (NYGLLYCFR)
a Not quantitated.
The values in parentheses are the number of residues based on the sequence.

TABLE II (Continued)

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES* OF 20K DALTON hGH SEPARATED BY RP-HPLC

Amino Acid	T-12	T-13	T-15	T-16	T-17	T-18 + T-19[θ]	Total
	126-130	131-143	153	153-163	154-163	164-168 + 169-176	
Asx		4.7(5)		1.8(2)	1.3(2)		20
Thr	0.9(1)	1.1(1)		1.1(1)	0.9(1)		10
Ser	1.0(1)	0.9(1)				1.4(2)	17
Glx	0.9(1)			1.2(1)	1.1(1)	2.0(2)	22
Pro							7
Gly						1.8(2)	8
Ala		1.1(1)					6
Cys						0.9(2)	4
Val				1.1(1)	0.9(1)	1.7(2)	7
Met				0.7(1)	0.8(1)		3
Ile						1.0(1)	7
Leu		2.0(2)		1.2(1)	1.0(1)		25
Tyr	1.1(1)						6
Phe		0.9(1)		1.0(1)	1.0(1)	0.9(1)	12
His		0.9(1)					3
Lys	0.8(1)	1.0(1)	1.0(1)	2.1(2)	0.8(1)		7
Arg				0.9(1)	0.9(1)	1.1(1)	11
Trp							1
Total Residues	149	162	163	163	163	176	176
Peak	3	11	1	13	15	11	

* Peptides are numbered in order of their position in the final sequence.
+ Amino acid sequence numbers
√ Not present in the Cys-(Cm)-20K-hGH digest, but replaced by peptide T4 (LHQLAFDTYQEFNPQTSLCFSESIPTPSNR) and peptide T-14 (NYGLLYCFR)
θ Not present in the Cys-(Cm)-20K-hGH digest, but replaced by peptide T-18 (IVQCR) and peptide T-19 (SVEGSCGF).
a Not quantitated.
The values in parentheses are the number of residues based on the sequence.

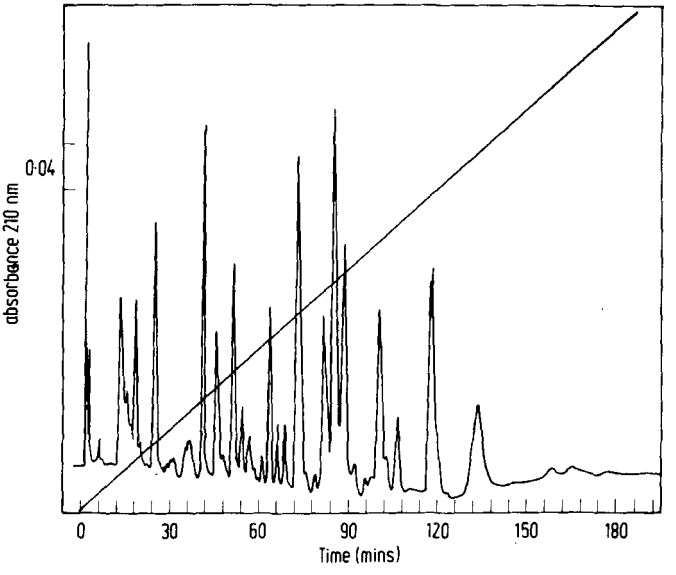

Figure 3. Separation of the tryptic peptides of human growth hormone (1400µg) on a Radial Pak A column at a flow rate of 1.5ml/min. using a 3-hour linear gradient generated from aqueous 100mM ammonium bicarbonate to 50% acetonitrile in 100mM ammonium bicarbonate.

hGH variant (T-1 to T-19) shown in Table II completely account for the intact 22K-hGH and 20K-hGH proteins respectively. Secondly, tryptic peptides T-1 to T-3, T-7 to T-13 and T-15 to T-21 of the 22K-hGH correspond in composition and retention characteristics to the tryptic peptides T-1 to T-3, T-5 to T-11 and T-13 to T-19 of the 20K dalton hGH variant. It is noteworthy that the tryptic peptides T-18 and T-19 of hGH (which correspond to the pair T-16 and T-17 of the hGH variant) are well resolved despite the single N-terminal lysine difference. These peptides, respectively (KDMDKVETFLR) and (DMDKVETFLR), correspond to residues 168-178 and 169-178 in the sequence of hGH. The relative elution order observed for these two peptides on the microparticulate octadecyl-silica with the low pH phosphate mediated elution conditions used is in accord with the known (21, 22) influence of an additional N-terminal lysine residue on peptide selectivity. Thirdly, identical chromatographic profiles were obtained for the corresponding peptides obtained from the digests of the Cys-(Cm) - proteins. In these cases, only peak 1 was shown to contain more than one tryptic peptide (corresponding to T-3, T-5 and T-17 of 22K-hGH). Under isocratic conditions with aqueous 50mM sodium dihydrogen phosphate, these peptides were not retained on the

μBondapak C_{18} column, but they can be resolved using a pairing ion elution system such as 15mM sodium heptane sulphonate, pH 3.0, (7, 21, 22) thus allowing unequivocal assignment for T-3 (AHR), T-5 (EQK) and T-17 (K). In the digest of the 20K dalton variant the peptide EQK (22K-hGH-T-5) is absent.

The results presented above indicate that portion of peptide T-4, peptide T-5 and part of peptide T-6 of hGH are not found in the 20K dalton hGH variant. These three peptides correspond to residues 20-38, 39-41 and 42-64 of the hGH sequence. Automated sequence analysis of this region of interest contained in peptide T-4 of the 20K dalton variant has shown that the partial sequence is LHQLAFDTYQEFNPQTSLC .... which corresponds in position to residues 20-31 and 47-53 of 22K-hGH. These results are consistent with the conclusion made in earlier studies by Lewis et al. (3) and Chapman et al. (4) that the N- and C- terminal sequences of the 20K dalton hGH variant are identical to the 22K dalton hGH form, but differs internally due to a 15 residue deletion corresponding to residues 32-46 of hGH.

Peak 5 shown in Fig. 2a eluted with a retention time of 6.7 min. and contained tryptic peptide T-14 of 22K-hGH. The compositional analysis of this peptide (Thr (0.9), Ser (1.0), Glx (0.7), Tyr (0.9) and Lys (1.0)) was in accord with values expected for a peptide with sequence QTYSK. Although the same peptide was present in the elution profile of the tryptic digest of the 20K dalton variant, an additional peptide (peak 3, Fig. 2b) of the same amino and composition, but shorter retention time (3.6 min.) was also obtained. The decreased retention on the octadecylsilica support shown by this peptide is consistent with a Gln → Glu change. The most plausible explanation for this observation is that the 20K dalton hGH variant, as isolated by the ion exchange procedure, contains material with either Gln or Glu at position 126 of 20K-hGH (position 141 on the hGH sequence). The origin of this difference is currently under investigation.

In summary, the separation of the tryptic peptides of the 22K dalton hGH and the 20K dalton hGH variant by RP-HPLC is described. By comparison with the known sequence of hGH, it was evident that all of the anticipated peptides were recovered thus permitting the sequence relationship between the 22K dalton and the 20K dalton hGH versions to be confirmed.

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