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Rapid verification of disulfide linkages in recombinant human growth hormone by tandem column tryptic mapping

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Abstract

An automated tryptic mapping method was developed for characterization of disulfide linkages in recombinant human growth hormone (rhGH). The hormone was trypsin digested and the peptide fragments concentrated by eluting rhGH through an immobilized trypsin column and transferring the peptides directly to a reversed-phase liquid chromatography (RP-LC) column where they were collected. Reaction time was controlled by the flow-rate. Following tryptic digestion of a sample, the immobilized enzyme column was uncoupled from the flow train by a switching valve and the RP-LC column eluted with a solvent gradient ranging from 0.1% trifluoroacetic acid (TFA) with 1% acetonitrile (ACN) to ACN with 0.1% TFA and 5% water. This two-step mapping process was achieved within 2 h on both native and reduced rhGH samples. The chromatographic elution position and mass spectra matrix-assisted laser desorption ionization time-of-flight mass spectrometry of native rhGH and sulfur-containing peptides were determined with standards. Standards of the individual sulfhydryl (–SH) containing peptides and all possible disulfide linked peptides that could result from coupling the –SH peptides in disulfide linkages were obtained by synthesis and chromatographic purification. This approach allowed the chromatographic elution position of all possible mismatched disulfide containing peptides to be established and samples of rhGH to be examined for improper folding. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Although large quantities of natural human proteins have been produced by recombinant DNA technology and used as therapeutic agents for several decades, confirming that the target protein has been biosynthesized and isolated without structural errors is still an issue. Genetic drift in the host organism, transcription errors, translation errors, variations in post-translational modification, oxidation, deamidation, proteolysis and disulfide mismatching during folding [1–3] have the potential to produce faulty structures. The concern is that these variant structures will be of reduced therapeutic efficacy or be immunogenic.

There are several ways the production of human therapeutic proteins could be regulated. One is by strictly regulating the process, as in the production of vaccines. The assumption in this mode of regulation is that a rigorously controlled process will always produce product identical to that used in the clinical efficacy trials. Another is by biological activity.

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Frequently the problem here is to find a suitable animal model, in addition to the ethical issue of sacrificing large numbers of animals in testing. Yet another is by structure. Because protein structure can be defined to the level of 1 Å in many cases, any of the structural errors noted above could be detected. Federal regulatory agencies have suggested that in future cases where the structure of human therapeutic proteins can be determined they might be regulated on the basis of structure. The problem with this approach is that more than a week can be required to totally elucidate the structure of a protein. This would contribute significantly to the cost of the therapeutic agent. The question is whether this level of structure proof is really necessary. There is a growing consensus that it is not. Shorter, quicker measurements which show (i) that product structure is consistent with the clinically tested material and (ii) that potential biosynthetic and process generated variants are absent will probably be the basis for future regulation.

There is now an intense effort to find rapid analytical methods for protein structure confirmation. Advances in matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) [4–7], electrospray ionization MS [6–9], capillary electrophoresis [10–12], liquid chromatography (LC) [13–15], and various combinations of these techniques [16–19] have accelerated protein characterization substantially. When instrumentation and the requisite chemical reactions involved in structure characterization are integrated into a single analytical system, there is the potential for automation. Automated affinity purification, proteolysis, tryptic mapping and mass spectral analysis of the tryptic peptides has recently been reported in the case of hemoglobin [20]. In another example, it has been shown that (i) proteins can be automatically reduced and alkylated in the autosampler of a liquid chromatograph, (ii) digested in an immobilized trypsin column in a few minutes, (iii) the tryptic fragments directly transferred to a reversed-phase liquid chromatography (RP-LC) column and (iv) the tryptic peptides chromatographically mapped [21]. These studies show that when the amount of immobilized trypsin on the column approaches or exceeds that of the target protein, digestion can be achieved an order of magnitude faster than in homogenous, solution based digestion. Other important advantages of the immobilized enzyme approach are that autodigestion of trypsin is diminished and reaction times are shorter, leading to cleaner digests and fewer structural artifacts from deamidation, oxidation and disulfide scrambling during proteolysis.

The focus of this work was to (i) exploit these new integrated multidimensional techniques for the analysis of disulfide linkages in recombinant human growth hormone (rhGH), (ii) confirm the presence of rhGH with the proper disulfide linkages and (iii) confirm the absence of improperly folded rhGH by synthesizing all possible mismatched peptide species and demonstrating their absence in tryptic digests.

2. Experimental

2.1. Instrumentation

Analyses were performed on either an Integral micro-analytical workstation or a BioCad liquid chromatography workstation (PerSeptive Biosystems, Framingham, MA, USA) in a tandem column configuration using absorbance at 215 nm to monitor eluents (Fig. 1). Proteolysis was achieved with a 100×2.1 mm Porozyme (PerSeptive Biosystems) trypsin cartridge. An LC-22A column heater (Bioanalytical Systems, West Lafayette, IN, USA) was used to maintain the temperature of the trypsin cartridge at 37°C during digestion. RP-LC separations were carried out on a 250×4.6 mm PepMap C_{18} column (PerSeptive Biosystems).

2.2. Chemicals and reagents

The research grade (i.e., not intended for human use) rhGH was a generous gift from Eli Lilly&Co. (Indianapolis, IN, USA). Synthetic human growth hormone (hGH) tryptic peptide analogues containing cysteine residues were provided by PerSeptive Biosystems. HPLC-grade acetonitrile (ACN) was a generous gift from EM Science (Gibbstown, NJ, USA). Dithiothreitol (DTT) and tris(hydroxymethyl)-aminomethane (Tris base) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade trifluoroacetic acid (TFA) was obtained from Pierce

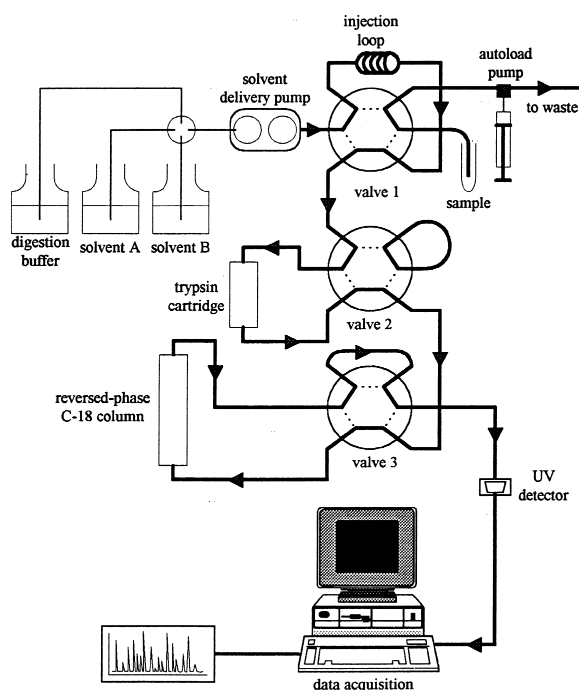


Fig. 1. Schematic diagram of the system used for on column tryptic digestion of recombinant human growth hormone.

(Rockford, IL, USA). Calcium chloride was purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.3. Solution preparation

Digestion buffer (0.05 *M* Tris, 0.01 *M* $CaCl_2$) was prepared by dissolving 6.06 g of Tris base and 1.11 g of calcium chloride in 1 l of double-deionized water and adjusting the pH to 8.0 with 6 *M* HCl. The solution was filtered through a 0.2-μm nylon 66 membrane filter (Alltech, Deerfield, IL, USA). Reversed-phase solvent A was prepared by adding 1 ml TFA and 10 ml ACN into 100 ml double-deionized water and bringing the total volume to 1 l. Solvent B was prepared by adding 1 ml TFA to 50 ml double-deionized water and adding ACN to a total volume of 1 l. All the digestion buffer and mobile phase solvents were degassed with helium before use.

2.4. Sample preparation

Solutions of 1.00 mg/ml and 2.00 mg/ml of rhGH were produced by dissolving the hormone in the proteolytic digestion buffer. Sample solutions were filtered with 0.25-μm poly(vinylidene difluoride) (PDFV) syringe filters (Alltech) before injected into a trypsin cartridge. Reduction of rhGH was achieved by adding dithiothreitol to the rhGH solution at a 20:1 molar ratio (DTT/rhGH) and incubating the mixture at room temperature for 30 min.

2.5. Tryptic digestion

An 80-μl volume of native rhGH solution (1.00 mg/ml) was injected into the trypsin cartridge at a flow-rate of 0.5 ml/min. After the sample entered the trypsin cartridge the solvent delivery pump was stopped and the sample was retained in the cartridge for 10~15 min. Flow was then resumed for 10 min to sweep the digested peptide fragments into the reversed-phase column. In the case of reduced rhGH, 40 μl of sample solution (2.00 mg/ml) with DTT (described in Section 2.4) were directly injected onto the trypsin cartridge. All other digestion conditions were the same.

2.6. Reversed-phase chromatography

Once the digested sample was transferred to the C_{18} reversed-phase column, the trypsin cartridge was switched off-line. The C_{18} column was then equilibrated with solvent A for 5 min at 1 ml/min. Subsequent to a 65-min gradient elution at 1 ml/min from solvent A to a 1:1 solvent A/B ratio, solvent composition was held at a 1:1 A/B solvent ratio for 5 min. Elution was monitored at 215 nm. Peptide peaks were manually collected, frozen in liquid nitrogen and lyophilized prior to MALDI-time-of-flight (TOF) MS analysis.

2.7. Disulfide bond pairing of synthetic hGH tryptic peptide analogues

Synthetic peptide analogues of the cysteine containing hGH tryptic fragments T6, T16, T20 and T21, were treated individually overnight at room temperature with a 20-fold molar excess of DTT in 100 m*M* Tris (pH 8.5) prior to purification with RP-LC. The individual purified peptides were manually collected, frozen in liquid nitrogen and lyophilized to dryness. The peptides were then reconstituted

in 100 m*M* Tris buffer (pH 8.5) to a final concentration of 0.4~0.9 mg/ml, mixed and exposed to oxygen for 2~4 h at room temperature. Under those conditions the peptides were oxidized and paired randomly to form disulfide linked peptides. A 40-μl volume of each reaction mixture was then chromatographed on a C_{18} reversed-phase column under the same gradient conditions described above.

2.8. Mass spectrometry

The lyophilized peptides were mixed with α-cyano-4-hydroxycinnamic acid (Sigma) which was dissolved in a mixture of ACN and 0.1% TFA. The matrix–analyte mixtures were analyzed by MALDI-TOF–MS on a Voyager Biospectrometry workstation (PerSeptive Biosystems).

3. Results and discussion

Disulfide linkages in a protein are frequently located by comparing the tryptic maps of reduced and unreduced samples. Cysteine-containing peptides in the reduced protein tryptic map are absent in the unreduced tryptic map; being replaced by a smaller number of new peaks consisting of two or more disulfide linked peptides. Peptide identification in

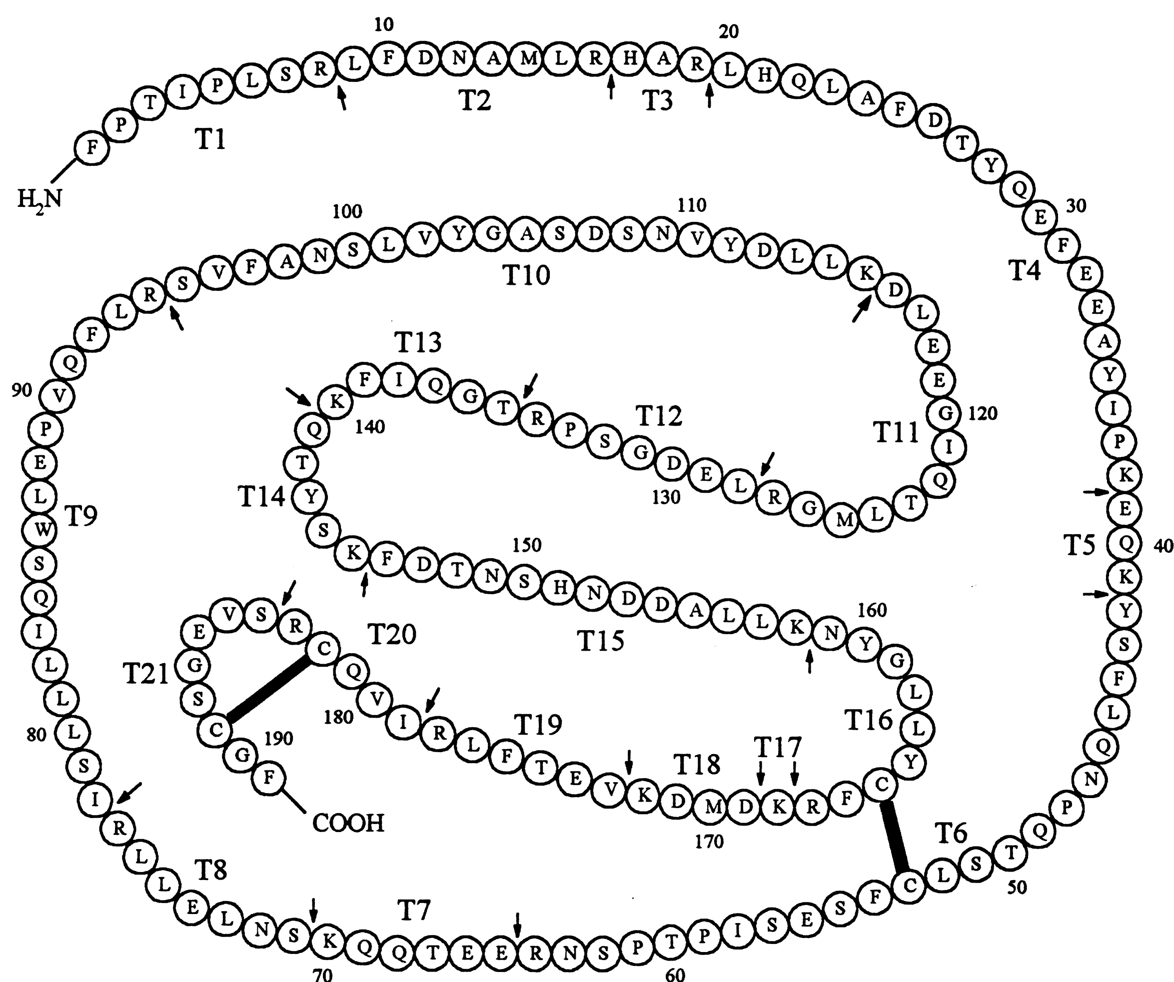


Fig. 2. Sequence representation of human growth hormone including the sites at which trypsin will theoretically cleave the protein (indicated by arrows) and the resulting tryptic fragments (indicated by T1, T2,... T21).

these disulfide linked species is generally achieved by (i) isolating and reducing the bridged species to release the individual peptide components for chromatographic identification or (ii) by direct mass spectral analysis of the unreduced peptide. Disulfide linked peptides frequently cleave in the mass spectrometer, yielding the component peptides which are identified by their molecular mass minus one atomic mass unit. Comparing with the separation behavior and/or mass of peptides in the reduced tryptic map allows location of disulfide linkages in the protein.

Because the objective in this study was to confirm the structure of a known protein and identify disulfide mismatches, a slightly different tactic was used. Cysteine-containing peptides in rhGH were synthesized and then oxidized to produce 10 possible disulfide linked species. Based on the chromatographic behavior of these disulfide linked standards, both native and mismatched disulfides were identified in tryptic digests.

A second objective of this study was to improve the speed of analysis by using immobilized trypsin columns in which the amount of trypsin approached or exceeded that of the protein substrate. This strategy differs from the conventional solution based total proteolysis in that the reaction time in the column is much shorter and proteolysis does not necessarily go to completion. It is to be expected in this kinetic approach that (i) the degree of proteolysis will vary substantially between proteins when using a fixed, short reaction time, (ii) incompletely digested fragments will be found at short

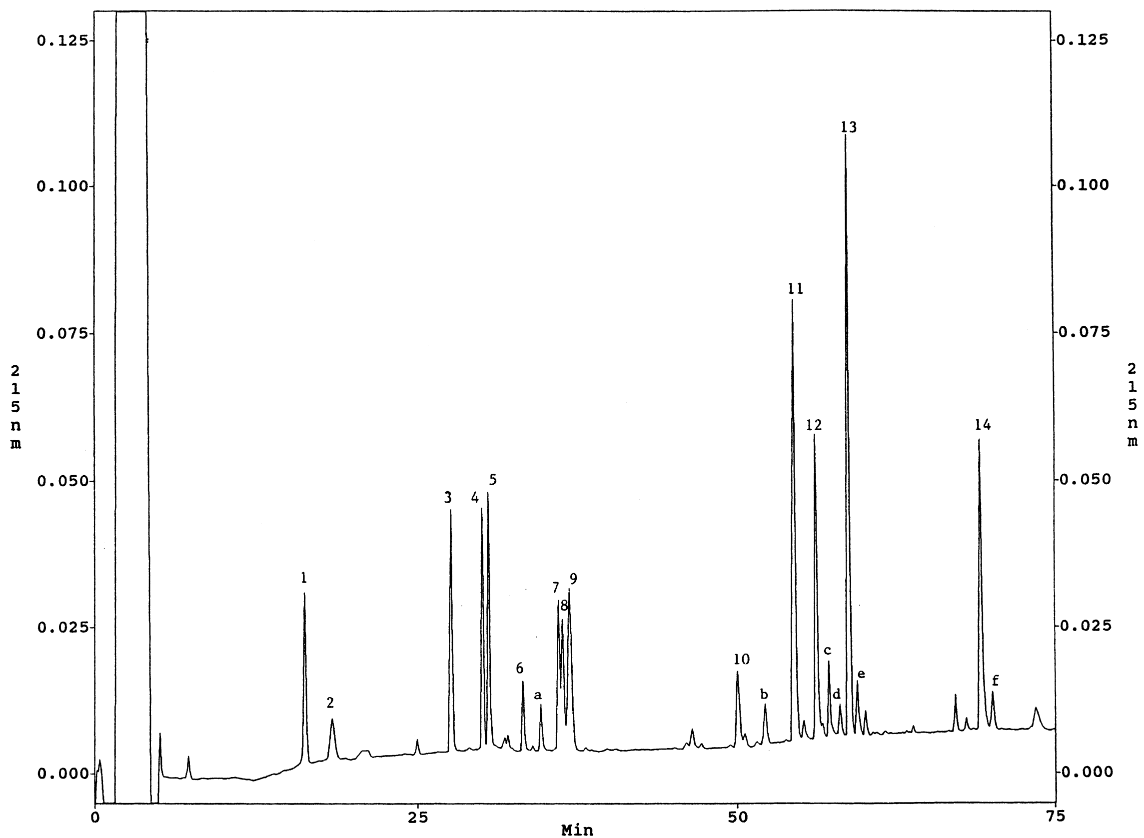


Fig. 3. Tryptic map of native recombinant human growth hormone. The digestion procedure and reversed-phase chromatography conditions are described in Sections 2.5 and 2.6. Tryptic fragments are labeled with numbers and the sequence assignment is given in Table 1. The peaks labeled with lower case letters correspond to incomplete tryptic digests: (a) T17+T18+T19; (b) T4+T5; (c) T6–16+T17; (d) T6–16+T7+T8; (e) T6–16+T15, and (f) T1+T2+T3+T4.

reaction times and (iii) the fragment ratio may not be the same as that found in solution based digestion.

3.1. On-column tryptic mapping of native rhGH

hGH has 11 arginine residues, 9 lysine residues and two disulfide linkages. Theoretically, trypsin digestion of unreduced rhGH should generate 19 fragments, two of which contain a disulfide bridge (Fig. 2). Tryptic mapping was achieved in a 2-h process with an integrated system where (i) tryptic digestion was executed in an immobilized enzyme column, (ii) the peptide fragments were transferred directly to a reversed-phase column for separation (Fig. 3) and (iii) the peaks were identified by MALDI-TOF–MS (Table 1). Four peptides, i.e., T3, T5, T7 and T17, were not found in the chromatogram. These peptides contain 1 to 6 amino acid residues and are thought to be sufficiently hydrophilic that they eluted unretained in the ''void'' peak. Peak 8 was found to be a peptide containing tryptic fragments T18 and T19. This indicates that the Lys–Val peptide bond in these two peptides was not cleaved by trypsin. Other peaks in the chromatogram labeled a, b, c, d and e were found to be due to incomplete tryptic digestion.

Typically, conventional tryptic digestion of hGH in solution requires 4–18 h [22–24]. In contrast, 50% digestion of rhGH was achieved in 10 min with on-column proteolysis (data not shown). The profile of the on-column tryptic map is very similar to those obtained from solution digestion [22–24]. This suggests that (i) the slowest step in trypsinolysis of rhGH is the initial cleavage of the protein, (ii) position specific differences in cleavage rates are small, with the exception of the Lys–Val bond connecting T18 and T19 and (iii) the rate of on-column tryptic digestion can be 20-times faster than in free solution. Acceleration of proteolysis in this case is obviously due to the fact that the trypsin/substrate ratio was 25:1 in the column whereas it is typically 0.02:1 or 0.001:1 in solution digestion. The large excess of immobilized trypsin creates an environment more kinetically favorable for the digestion of rhGH.

3.2. On-column tryptic mapping of reduced rhGH

Native, unreduced rhGH contains two disulfide linkages, one between Cys53 and Cys165 and the other between Cys182 and Cys189. When rhGH is reduced with dithiothreitol and digested with trypsin, Cys53 appears in the tryptic map (Fig. 4) in peptide T6, Cys165 is in peaks T16, Cys182 is located in T20, and Cys189 is in peak T21. These four new peptides, T6 (eluting at 57.2 min), T16 (53.7 min),

Table 1
Retention time, mass and peak assignments of peptides from on column tryptic mapping of unreduced rhGH

Peak No.	Retention time (min)	Mass (u)		Peak assignment
		Theoretical	Observed	
1	16.2	626.7	625.2	T14
2	18.4	772.8	774.2	T12
3	27.5	693.8	692.3	T13
4	29.9	1400.5	1402.5	T20–21[a]
5	30.4	1487.5	1486.9	T15
6	33.1	844.0	844.7	T8
7	35.9	979.1	978.4	T2
8	36.2	1254.4	1259.1	T18+10[b]
9	36.9	933.1	933.6	T1
10	50.0	1359.5	1361.8	T11
11	54.4	2340.5	2341.2	T4
12	56.2	2261.4	2260.9	T10
13	58.8	3767.1	3754.8	T6–16[a]
14	69.3	2056.5	2053.6	T9

[a] Disulfide bond.
[b] Lys–Val bond (not cleaved).

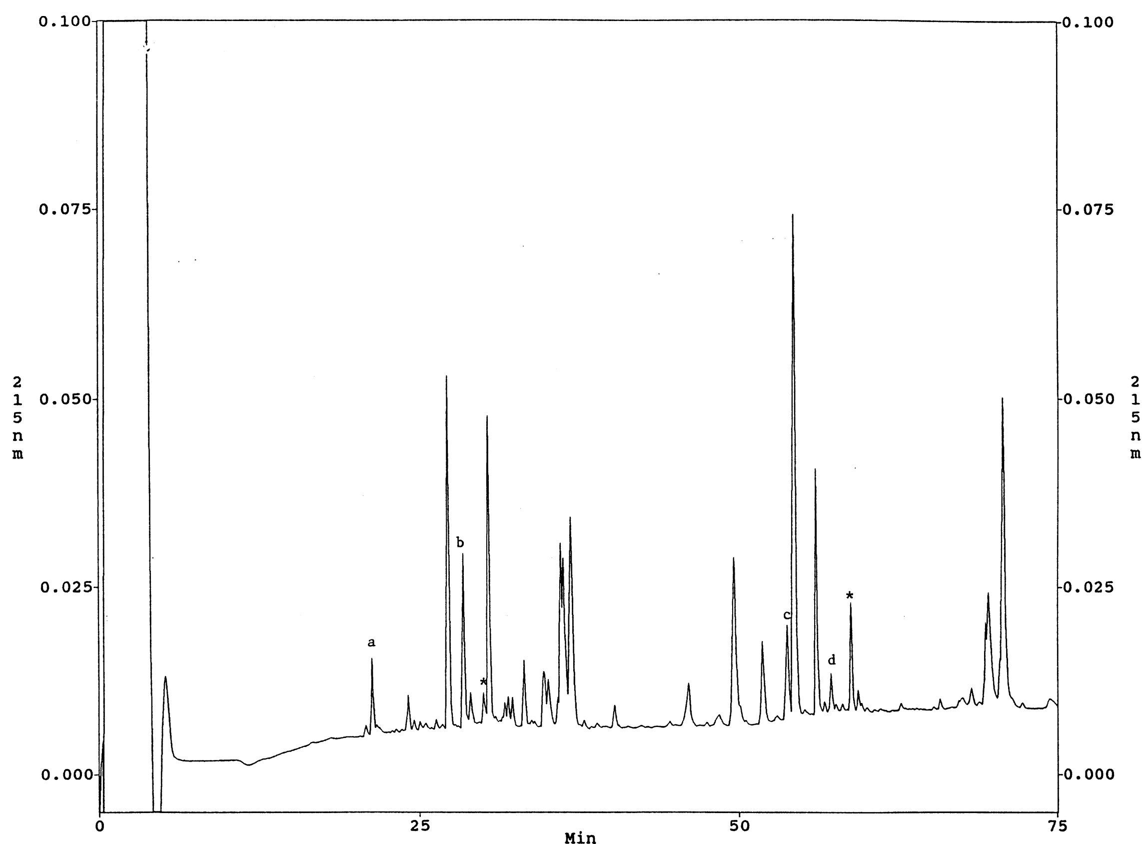


Fig. 4. Tryptic map of reduced recombinant human growth hormone. The protocol and conditions for reduction, digestion and reversed-phase chromatography are described in Section 2.4 Section 2.5 Section 2.6. The peaks labeled with lower case letters are (a) T20; (b) T21; (c) T16 and (d) T6, respectively. The peaks labeled with an asterisk correspond to unreduced disulfide bridged T20–21 and T6–16.

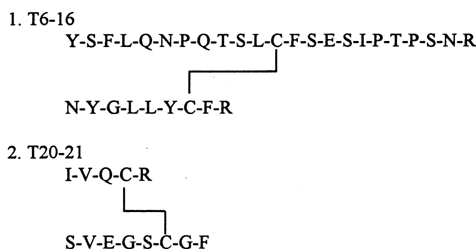
T20 (21.3 min) and T21 (28.4 min), replace the two disulfide bridged peptides T6–16 and T20–21 in native rhGH tryptic map (Fig. 3). Peak identification is based on comparisons of the chromatographic and mass spectral properties of these peptides with those of synthetic peptides of known sequence. It is noticed in the chromatogram of reduced rhGH that peaks corresponding to T20–21 (29.9 min) and T6–16 (58.8) appear at low levels. This suggests that the disulfide bonds were not fully reduced by DTT under the conditions used.

The objective in this protocol was to not alkylate disulfides on the reduced protein by quenching the DTT reducing agent with an alkylating reagent. It should be noted that direct injection of DTT into the immobilized trypsin columns will diminish column lifetime by reducing the enzyme and slowly causing irreversible denaturation [21]. Because direct injection of DTT is rarely necessary in this method, immobilized enzyme columns lasted 3–6 months.

3.3. Disulfide bond pairing in synthetic hGH peptides

The possibility of non-native disulfide bond formation either during biosynthesis or processing was explored in this work. On a theoretical basis, random disulfide bond formation between the four cysteine residues in human growth hormone could generate native rhGH in addition to four mispaired intramolecular disulfide linked species (Fig. 5). Synthetic T6, T16, T20 and T21, each containing a cysteine residue, were used to generate a series of disulfide linked (–S–S–) peptides. The retention time and

A. Correct disulfide linkages in rhGH



B. Possible mismatched intra-molecular disulfide linkages

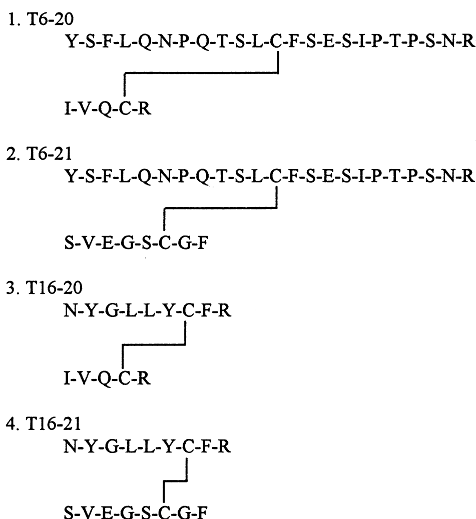


Fig. 5. Possible intra-molecular disulfide linked species from human growth hormone. (A) Two correct structures and (B) four mismatched structures.

mass of each synthetic –S–S– peptide was compared with peptides from rhGH samples (Table 2). Retention times of the synthetic –S–S– peptides T20–21 (30.0 min) and T6–16 (59.4 min) correspond closely with those obtained from on-column tryptic mapping (29.9 and 58.8 min, respectively). The small difference in retention time is believed to be due to variance in gradient generation. Retention times of the four peptides with mismatched disulfide linkages are 45.8 min (T16–20), 51.6 min (T6–20), 55.2 min (T6–21) and 57.2 min (T16–21), respectively. Examination of the chromatograms from tryptic mapping of native and reduced rhGH reveals that a peak with a retention time of 57.2 min is present in both the native and reduced rhGH tryptic maps. Mass spectra (Fig. 6) of these peaks reveal

Table 2
Retention time and mass of disulfide-linked synthetic hGH peptide analogues

Disulfide linkage	Retention time (min)	Mass (u)	
		Theoretical	Observed
T6–16[a]	59.4	3767.1	3762.4
T20–21[a]	30.0	1400.5	1401.1
T6–20[b]	51.6	3236.6	3235.1
T6–21[b]	55.2	3401.6	3402.1
T16–20[b]	45.8	1766.1	1764.2
T16–21[b]	57.2	1931.1	1935.0

[a] Correct disulfide linkage.
[b] Mismatched disulfide linkage.

different fragment ions for native and reduced rhGH. The mass spectrum indicates that the chromatographic peak from the native rhGH tryptic map is comprised mainly of the peptides T6–16 and T17 (a lysine residue). In addition, a very small amount (estimated to be less than 1% from the mass spectrum) of a peptide with $M+H^{+}=1943.4$ was present. This is close to the theoretical mass value of T16–21 (Table 3) but because (i) the mass deviation between the unknown and T16–21 is slightly greater than 0.5% and (ii) MALDI-TOF mass spectrometers are capable of detecting mass differences at this molecular mass of $<$0.5% deviation, it can be concluded that this substance is not a mismatched, disulfide linked peptide.

In the case of reduced rhGH, the mass spectrum of the peak at 57.2 min shows that it contains multiple peptide fragments. Among them were small amounts of two fragments with masses that correspond to T16–21 and T6–6, respectively. The only way these can form is either from a disulfide crosslinked dimer or from reoxidation during proteolysis and analysis. The former is unlikely because dimer was not found in the sample by either MS or size-exclusion chromatography (data not shown). Reoxidation during analysis is much more likely. It is known that reduced rhGH undergoes reoxidation easily under slightly alkaline conditions [25,26]. Furthermore, similar conditions were used to synthesize the disulfide linked peptides used in these studies. It is concluded that T6–6 and T16–21 in this case were most likely formed at the inlet of the chromatography column where the cysteine peptides were exposed to incompletely degassed, oxygen contain-

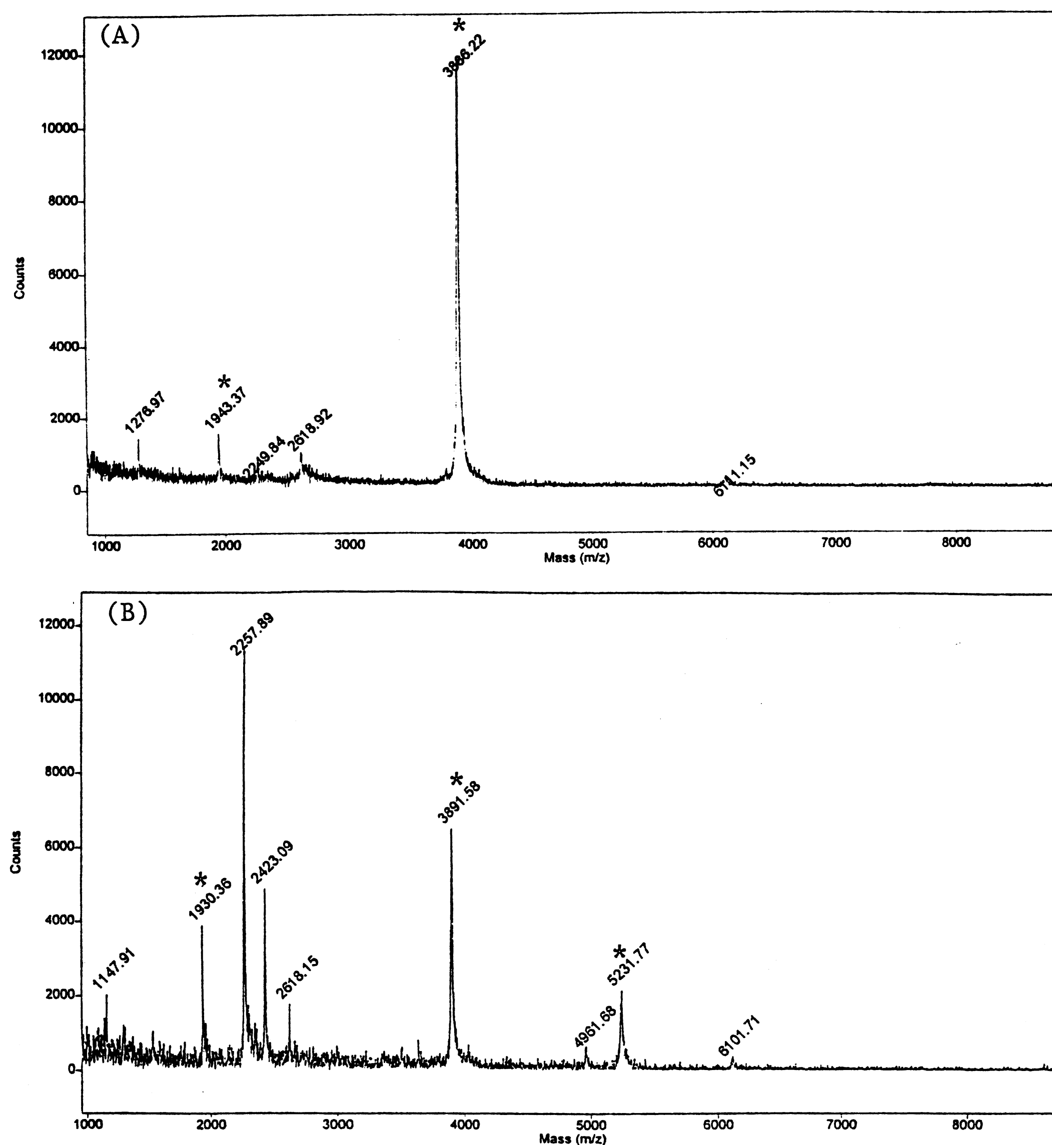


Fig. 6. Mass spectra of peptide fragments eluting at 57.2 min. (A) Native rhGH and (B) reduced rhGH. Explanations for the peaks labeled with an asterisk are given in Table 3.

ing mobile phase before they were separated. These small amounts of disulfides in the reduced samples are probably artifacts that can be eliminated by more rigorous degassing of the mobile phases. It should be noted that this phenomenon was not observed in non-reduced samples, i.e., T6–6 was never observed in a non-reduced tryptic map of rhGH. This is important because it diminishes the possibility that disulfide scrambling occurs during the analysis of disulfide linked peptides.

3.4. Other proteins

Proteins vary enormously in both amino acid composition, structural properties and response to analytical methods. In the specific case of the

Table 3
Mass analysis and percentage error of peptide fragments from native rhGH and reduced rhGH tryptic maps that eluted with a retention time of 57.2 min

	Retention time	Possible S–S linkage	Mass (u)		Error (%)
			Theoretical	Observed	
Unreduced rhGH	57.2	T6–16+17	3892.2	3886.2	0.15
		T16–21	1931.1	1943.4	0.64
Reduced rhGH	57.2	T6–16+17	3892.2	3891.7	0.02
		T16–21	1931.1	1930.4	0.04
		T6–6	5237.6	5231.8	0.11

procedure described above for location of disulfide bridges in hGH, it will probably require modification for some other proteins or may not apply at all. Some proteins are digested very slowly by trypsin in the native state. Using immobilized trypsin columns with an enzyme to substrate ratio orders of magnitude higher will greatly accelerate proteolysis, but hours of digestion may still be required. For other proteins it is essential to block the disulfides to prevent reoxidation. Still other proteins require a reduction in pH to prevent disulfide reshuffling. None of these variants was examined in this work. Although immobilized proteolytic enzymes can be a power analytical tool in protein characterization, it is unlikely that any single proteolytic method will be universal.

4. Conclusions

It may be concluded that an integrated system in which trypsin digestion and RP-LC are performed in tandem columns allows the position of disulfide linkages in rhGH to be confirmed an order of magnitude faster than with existing methods. This method will be particularly valuable where it is the objective to confirm that (i) protein folding based on disulfide bond formation has occurred correctly and (ii) mismatching to as low as 1% may be detected when MS is used to look for mismatched species. In addition, chromatographically based tryptic maps of non-reduced rhGH contain information to simultaneously confirm more than 90% of the sequence during the course of disulfide linkage analysis. Finally, it is probable that when coupled with MS to detect both peptides eluting in the chromatographic void volume and minor species coeluting with major peptide fragments that it will be possible with rhGH to determine in 2 h (i) greater than 95% of the primary structure and (ii) the position of all disulfide linkages in the protein sample to the level of 1%. If rhGH is similar to other proteins, then this is generally true.

Acknowledgements

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