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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PEPTIDE MAPPING OF BOVINE SOMATOTROPIN*

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SUMMARY

Reversed-phase high-performance liquid chromatography peptide mapping techniques have been used to examine the primary structure of bovine somatotropin (bSt) isolated from *Escherichia coli* modified by recombinant DNA techniques (rbSt) and from bovine pituitary (pbSt). Peptide fragments arising from tryptic digestion of bSt were separated on Baker wide pore C_4 or C_8 columns with linear gradients (acetonitrile–water with 0.1% trifluoroacetic acid). Major peaks eluted in a consistent order, but significant day-to-day variation in retention times was observed. Isolated peptide fragments were analyzed via acid hydrolysis followed by formation and separation of the phenylthiocarbamyl amino acids. These correspond to expected tryptic fragments.

INTRODUCTION

Peptide mapping techniques have been used by many researchers to examine the primary structure of proteins. A protein is selectively cleaved via chemical or enzymatic reactions and the resulting peptides are separated and subsequently analyzed. Agents commonly used for selective cleavage include trypsin, chymotrypsin, pepsin and cyanogen bromide[1]. Peptide separations have been performed by thin-layer chromatography (TLC), electrophoresis and high-performance liquid chromatography (HPLC)[2–5]. Analysis of the peptides often includes determination of amino acid composition via hydrolysis and subsequent chromatographic assay[6] and determination of sequence[7].

A need for a peptide mapping method arose out of efforts to produce bovine somatotropin (bSt) from *Escherichia coli* modified by recombinant DNA techniques (rbSt). The native protein has been previously isolated from bovine pituitary (pbSt) and mapped[2].

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We chose trypsin as our cleavage agent because of its specificity for cleaving the carboxyl side of peptide bonds of lysine and arginine residues[8] and because of previous uses in other peptide mapping studies[2–5].

Reversed-phase HPLC (RP-HPLC) using wide pore silica and perfluorinated acids as solvent modifiers was chosen as the method for separating tryptic peptides due to its well documented resolving power and speed[9–11]. Amino acid analysis via the phenylthiocarbamyl derivatives was chosen due to its speed and sensitivity[12,13].

EXPERIMENTAL

Materials

UV grade acetonitrile (Burdick & Jackson, Muskegon, MI, U.S.A.) dithiothreitol (DTT) and HPLC spectro-grade trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.), TPCK [L-(tosylamido-2-phenyl)ethylchloromethyl ketone] treated trypsin lot 33H792 (Worthington Freehold, NJ, U.S.A.), analytical grade reagents and distilled, deionized MilliQ™ water (Millipore, Bedford, MA, U.S.A.) were used. Samples of rbSt were obtained from Upjohn sources and pbSt was obtained from A. F. Parlow (Harbour Medical Center, UCLA, Los Angeles, CA, U.S.A.).

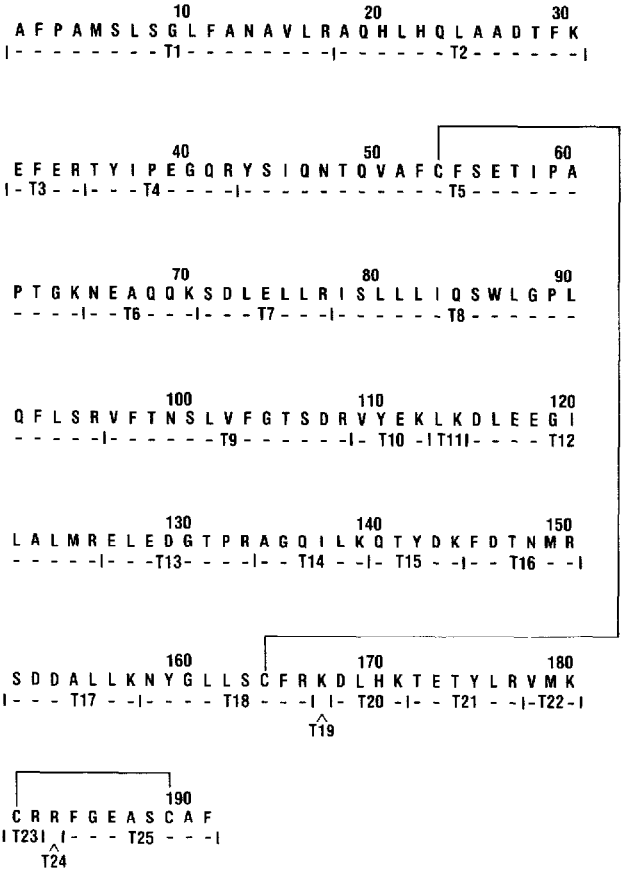

Fig. 1. Expected tryptic peptides from bSt. Solid lines indicate disulfide bonds.

Chromatography

Various combinations of the following devices were used: wide pore WP C_4 and C_8 250 mm × 4.6 mm I.D. columns (J. T. Baker, Philipsburg, NJ, U.S.A.), Series 4 solvent delivery system, ISS-100 autosampler and LS-4 fluorescence detector (Perkin Elmer, Norwalk, CT, U.S.A.), 1090 liquid chromatograph and 1040A diode array detector (Hewlett Packard, Palo Alto, CA, U.S.A.), Model 840 data station and PICO-TAG™ amino acid analysis system (Waters Assoc., Milford, MA, U.S.A.), Vista 5500 data system (Varian, Walnut Creek, CA, U.S.A.), Spectroflow 773 absorbance detector (Kratos, Ramsey, NJ, U.S.A.) and UV III monitor 1203 with 214-nm kit (LDC, Riviera Beach, FL, U.S.A.).

Trypsin digestion

The effects of bSt concentration, buffer concentration, trypsin–bSt ratio, and digestion time and temperature were examined. The digestion sequence used for routine mapping was conducted as follows: bSt was dissolved in 0.1 *M* ammonium bicarbonate buffer (pH 7.8) to make about 200 μg bSt/ml. Trypsin was added to give a trypsin–bSt ratio of 1:20 (w/w) and the digestion mixture was incubated at room temperature for at least 16 h. The digest was split into two equal portions. A volume of TFA solution was added to one portion to give *ca.* 1% TFA and quench the reaction. This was designated the oxidized (OX) digest. The other portion was mixed

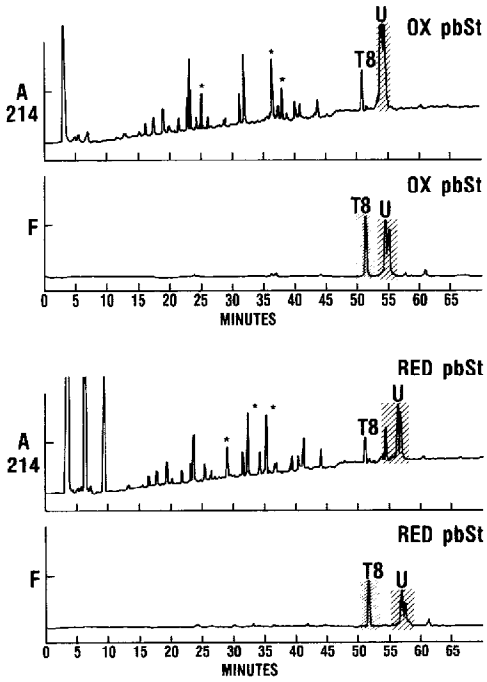

Fig. 2. Chromatograms showing incomplete digestion and comparing detection at 214 nm to fluorescence (excitation, 280 nm; emission, 340 nm). Gradient, 0–60% B in 60 min on WP C_4 (A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile). T8 = T8 fragment containing Trp (W); U = partially digested bSt; ★ = peaks which are different in OX and RED preparations.

with 10 mg/ml DTT in 0.1 *M* ammonium bicarbonate to give *ca.* 100 molar excess of DTT. The reduction was allowed to proceed for 1 h at room temperature and it was then quenched with TFA as above to give the reduced (RED) digest.

RP-HPLC

Various combinations of columns, mobile phases, gradient slopes and flow-rates were examined. Conditions for experiments are listed in figure captions and described below.

Amino acid analysis

Tryptic peptides isolated by RP-HPLC were lyophilized, hydrolyzed under acidic conditions and the resulting amino acids quantitated via phenylthiocarbamyl (PTC) derivatives using the Waters PICO-TAG™ system[12,13].

RESULTS AND DISCUSSION

Trypsin digestion

Fig. 1 shows a schematic of the expected tryptic fragments of bSt. The results of the optimization of the digestion are summarized below.

TABLE I

SUMMARY OF AMINO ACID ANALYSIS OF THE OX TRYPTIC MAP OF rbSt. SEE UPPER CHROMATOGRAM IN Fig. 5

T = Theory; R = recovered.

Amino acid	*Retention time (min)*																			
	72.8		*40.5*		*17.6*		*25.6*		*63.5*		*6.2*		*44.1*		*86.6*		*48.0*		*11.1*		*67.8*	
	Tryptic fragment No.																					
	T1		*T2*		*T3*		*T4*		*T5+18*		*T6*		*T7*		*T8*		*T9*		*T10*		*T11+12*	
	T	*R**	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R***	*T*	*R**	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*
Asp	1	1.0	1	1.2					2	2.4	1	0.7	1	1.2			2	2.4			1	1.3
Glu			2	2.1	2	1.6	2	2.3	3	3.0	3	2.5	1	1.0	2	1.7			1	0.8	2	2.3
Ser	2	2.0							3	3.3			1	0.9	3	2.6	2	2.0				
Gly	1	1.0					1	0.9	2	2.2					1	1.0	1	1.0			1	1.1
Hls			2	2.0																		
Arg	1	1.3			1	1.0	1	1.0	1	0.9			1	1.0	1	1.0	1	1.0			1	1.5
Thr			1	0.8			1	1.0	3	2.3							2	1.9				
Ala	4	3.6	3	3.2					2	1.9	1	1.0									1	1.1
Pro	1	1.0					1	0.9	2	1.8					1	0.9						
Tyr							1	0.9	2	1.6									1	0.9		
Val	1	0.7							1	0.9							2	1.5	1	0.9		
Mat	1	0.7																			1	0.8
Cys									2	0.3												
Ile							1	1.5	2	1.5					2	1.5					1	0.8
Leu	3	2.8	2	2.1					2	2.0			3	2.7	6	4.9	1	1.0			4	4.1
Phe	2	1.9	1	1.1	1	0.9			3	2.4					1	0.9	2	1.6				
Lys			1	1.0					1	1.0	1	1.4							1	1.0	1	1.0

* Based on a residue other than Arg or Lys.

** Adjusted for contamination by R1-14 (= peptide containing residues 1-14 which contaminated this fraction).

Buffer concentration

Trypsin exhibits maximum enzymatic activity around pH 7.8–8.0 and 0.1 *M* ammonium bicarbonate (pH 7.8) has commonly been used[2,5,8]. The 0.1 *M* concentration was also found to be optimum for bSt as 0.01 *M* resulted in incomplete digestion and 1.0 *M* caused precipitation of the peptides. Fig. 2 shows chromatograms obtained for an incompletely digested preparation. Note the presence of a number of late eluting peaks with strong fluorescence. These are believed to be due to partially digested bSt fragments which contain the T8 peptide. The T8 fragment contains the only tryptophan (Trp) residue in bSt and thus is the only fragment peak which exhibits strong fluorescence (peak at about 51 min in Fig. 2). Peptide fragments containing tyrosine (Tyr) or Trp also exhibited absorbance at 280 nm (not shown in figures). Peaks which had 280 nm absorbance were assigned as T4, T5, T10, T15, T18 and T21 with T5 bridged to T18 in the OX digest by a disulfide bond (see Table I and Fig. 5).

Protein concentration

A concentration of 200 μg/ml bSt was found to be optimum for digestion and subsequent detection of the peptide fragments. Solubility problems were encountered above 500 μg/ml and sensitivity problems in the HPLC analysis were observed below 100 μg/ml.

67.4		*21.3*		*24.2*		*34.0*		*27.1*		*29.6*		*12.0*		*11.7*		*26.5*		*9.7*		*36.2*		*37.7*	
T12		*T13*		*T14*		*T15+16*		*T16*		*T17*		*T19+20*		*T20*		*T21*		*T22*		*T23+25*		*T23+25*	
T	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R**	*T*	*R*
1	1.3	1	1.5			3	3.1	2	1.9	2	2.6	1	1.0	1	0.8								
2	1.9	2	2.3	1.1.3	1	0.9									1	.2			1	1.0	1	1.0	
										1	1.0									1	1.0	1	1.2
1	1.1	1	0.9	1	1.0															1	1.0	1	1.1
												1	0.9	1	1.0								
1	1.0	1	1.0			1	1.0	1	1.0							1	1.0			1	1.4	2	2.0
		1	1.0			2	1.7	1	1.0							2	2.1						
1	1.1			1	1.0					1	1.0									2	1.7	2	1.9
		1	0.9																				
						1	0.9									1	1.0						
																		1	1.1				
1	0.5					1	0.7	1	0.6									1	0.8				
																				2	0.4	2	0.2
1	0.8			1	0.9																		
3	2.7	1	0.9	1	0.9					2	2.0	1	0.9	1	0.9	1	0.9						
						1	0.8	1	0.8											2	1.7	2	2.0
				1	1.0	1	0.9			1	1.0	2	2.0	1	1.0			1	1.0				

Trypsin–bSt ratio

Enzyme to hormone ratios ranging from 1:20 (w/w) to 1:200 (w/w) have been reported in the literature for tryptic digestion[2,5,8]. Complete removal of the intact bSt and "core" peptide peaks due to digestion were observed in the range of 1:20 to 1:40 for trypsin–bSt. No interference due to trypsin was observed, thus 1:20 (w/w) was selected to give maximum tryptic activity.

Digest time and temperature

No significant difference between digests performed at 37°C or room temperature were observed. Incomplete digestion was observed after 6–8 h, therefore, an overnight incubation was required. No significant differences between 16 and 24 h were observed. The addition of TFA quenches the enzymatic activity and stabilizes the peptide fragments.

Reduction

DTT is commonly used to reduce disulfide bonds. It is most effective around pH 8, therefore the same 0.1 *M* ammonium bicarbonate buffer was used. Reduction reactions with DTT are complete within 1 h at room temperature and a one hundred fold excess on a molar basis is commonly used[14]. Again, TFA is used to quench the reaction and stabilize the sample preparations.

Sample stability

Tryptic digests quenched with TFA showed no significant change in the peptide maps after three days at room temperature.

RP-HPLC systems studied

The columns studied included Waters μBondapak C_{18}, Hibar LiChrosorb RP-18, DuPont Zorbax C-8, Zorbax 150 Å C-8, Zorbax PEP-RP1, and Baker Bond wide pore series of C_4, C_8 and diphenyl. A linear water–acetonitrile gradient using 0.1% TFA was used in each case. The Baker Bond wide pore alkyl C_4 and C_8 columns provided superior performance and were selected for further investigation.

The ionic mobile phase modifiers studied included 0.1% TFA, 0.1% orthophosphoric acid, 0.1% heptafluorobutyric acid (HFBA), various phosphate buffers, and ammonium bicarbonate. Solubility problems with bSt were encountered in the pH 3–8 range, thus the acidic modifiers were pursued. Various combinations of triethylamine or morpholine with either TFA or orthophosphoric acid were studied. The amines demonstrated no effectiveness in reducing tailing and enhancing peak shape. Both 0.1% orthophosphoric acid and 0.1% TFA provided similar results in terms of peak sharpness. TFA was selected because at pH 2.2 it is slightly less harsh on the column than the pH 1.9 phosphoric acid.

The strong solvents investigated included acetonitrile, methanol and *n*-propanol. Linear gradients were run in each case. Solubility problems were encountered with methanol. The *n*-propanol is a considerably stronger solvent for RP-HPLC than acetonitrile and was not suitable for the digest chromatography.

Gradient profile

A linear gradient of 0–70% acetonitrile in 70 min (1%/min) with a flow-rate

of 1 ml/min was used in the initial development work to ensure elution of the peptide fragments and undigested bSt if present (see Fig. 2). Subsequent work determined that all of the tryptic peptides elute with 45% acetonitrile. In addition, superior resolution and definition of the peptide peaks was observed with a gradient slope of 0.5%/min and a flow-rate of 1.5 ml/min. The use of shallow gradient slopes and increased flow to enhance resolution is consistent with other reports in the literature[9,10,15]. The linear gradient profile of 0–45% acetonitrile in 90 min was found suitable with each of the column packing types (C_4 and C_8) studied.

Reproducibility and ruggedness

This is probably the most interesting yet puzzling aspect of the peptide mapping chromatographic method. Very good reproducibility is observed for the retention times of the various peaks within a given run on the same column on the same day [relative standard deviation (R.S.D.) less than 2%]. Six replicate digests of rbSt were prepared and chromatographed in the same run. This HPLC reproducibility is illustrated in Fig. 3 for the oxidized and reduced preparations respectively. The two

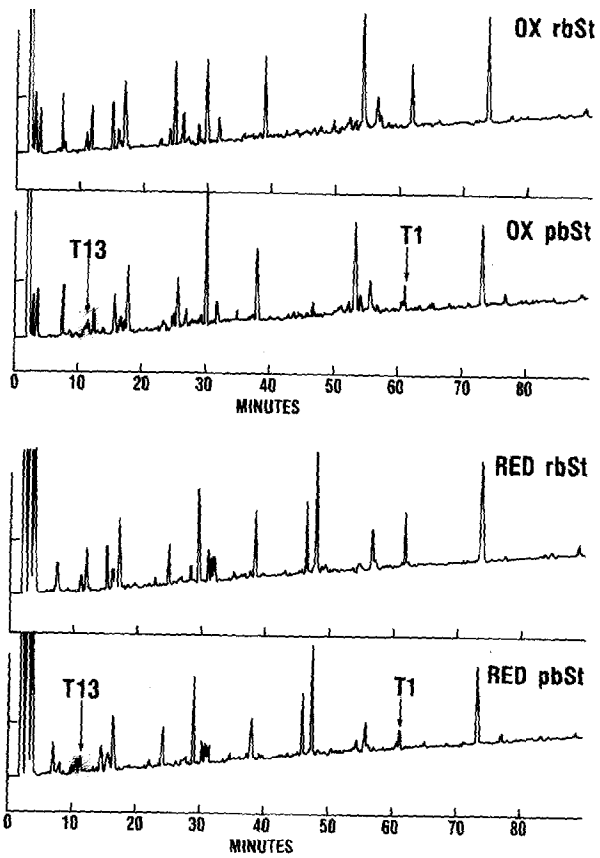

Fig. 3. Comparison of tryptic digests of rbSt and pbSt. Note the multiplicity of peaks in the T13 and T1 regions in pbSt. Gradient, 0–45% B in 90 min on WP C_4 (A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile). Detection at 214 nm, *ca.* 0.1 a.u.f.s. Injected *ca.* 200 μl.

additional large peaks in the reduced samples are due to the presence of DTT. These data indicate that a given gradient system is quite reproducible and rugged within a run. In addition, only minor deviations in retention behavior are observed with different batches of mobile phases. However, when individual columns of the same type (*i.e.*, 25 cm WP C_4) are compared, significant differences in retention behavior are observed as illustrated in Fig. 4 for the chromatograms of oxidized preparations. The peak elution order remains fairly consistent, but a significant variation in capacity factor (k') values is observed. This variability was observed with the C_4 and C_8 packings even when the digests were run using identical instrumental conditions. Both packings provided similar elution patterns with a large variability in k'. For these reasons it is necessary to prepare and run a reference material for comparison with each set of samples. A broad tailing artifact peak eluting in the region of 35–40 min is also observed in some of the chromatograms in Fig. 4. The position and intensity of this artifact peak varied from run to run. It appears to be associated with incomplete degassing of the mobile phase.

Data interpretation

The primary structure of pbSt[2] is illustrated in Fig. 1. The 25 peptide fragments predicted from enzymatic cleavage with trypsin are labelled T1 through T25. Four of those fragments are not retained and consequently are not separately detected with

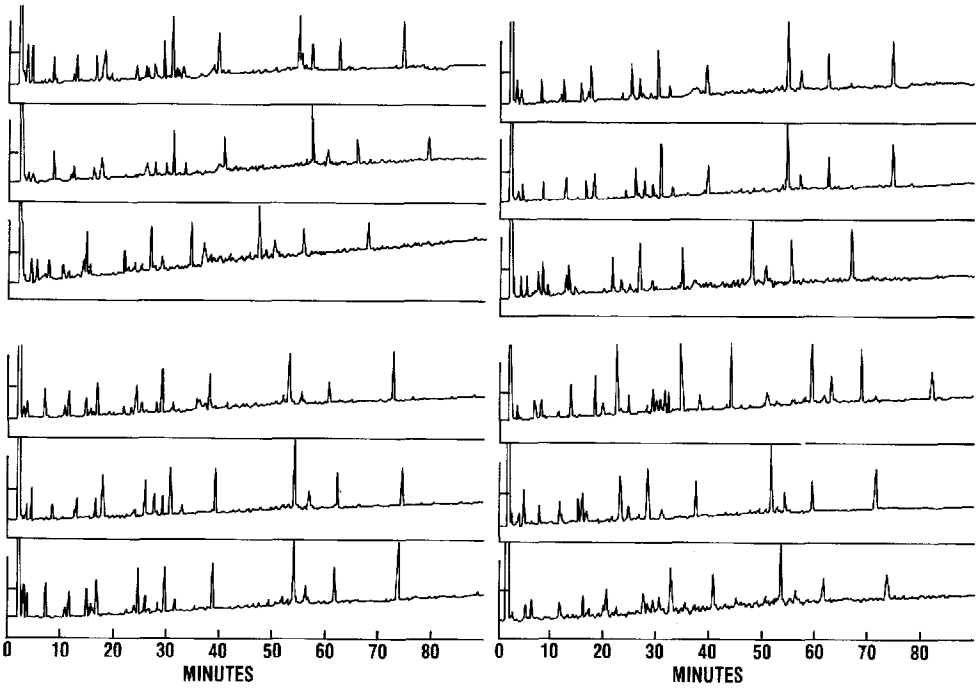

Fig. 4. Example of observed column-to-column variation in chromatography. Note major peaks elute in the same order. Within a run retention times of individual peaks varied less than 2% (R.S.D.). Gradient, 0–45% B in 90 min on WP C_4 (A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile). Detection at 214 nm, 0.1 a.u.f.s. Injected *ca.* 200 μl.

this chromatography as two (T19 and T24) are single amino acids and two (T11 and T23) are dipeptides. The remainder of the fragments comprise the expected peptide map for bSt.

Representative peptide mapping chromatograms for the oxidized and reduced sample preparations of rbSt are shown in Fig. 5 with tentative peak identification. These peak assignments are based on amino acid analysis of isolated fragments. This was supplemented with information obtained by comparison of tryptic digests of bSt fragments prepared via cyanogen bromide digestion of S-alkylated pbSt[16,17] and also based on another report on rbSt mapping[18]. These peak identifications will be confirmed with sequence analysis of the isolated tryptic fragment peptides.

However, with these tentative peak assignments several observations on key features of the peptide maps can be made. The last eluting peak has been identified as T8. It contains the only tryptophan residue in bSt and possesses very strong fluorescence (excitation 280 nm; emission, 340 nm) (Fig. 2). Differences between oxi-

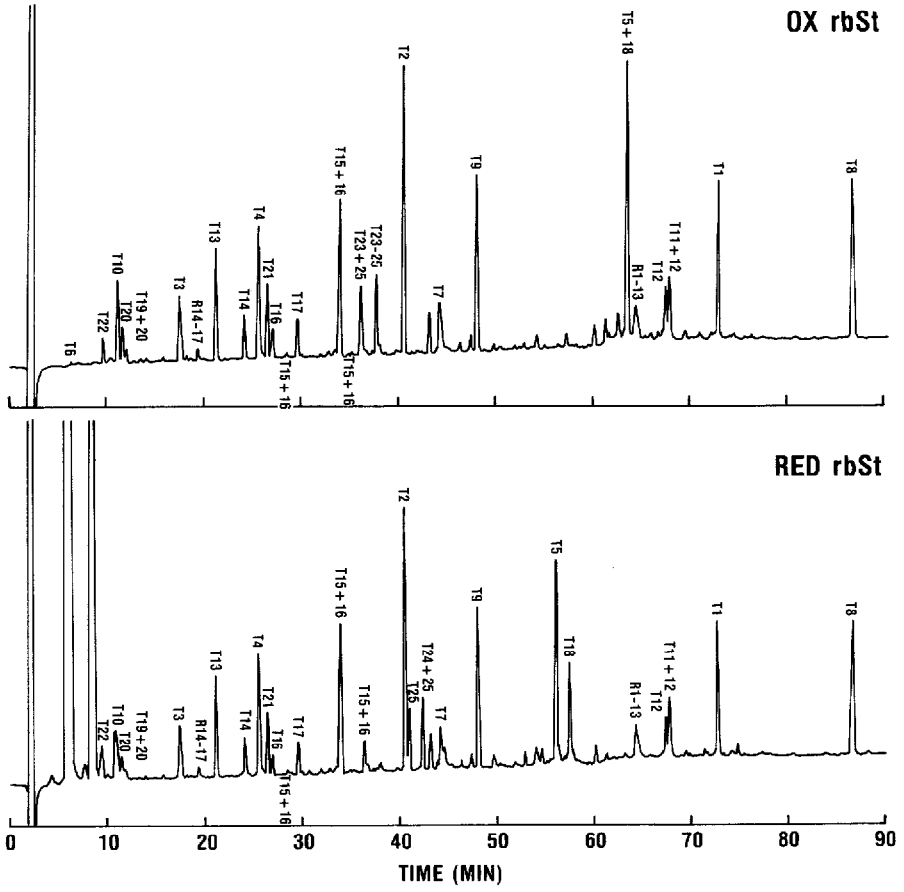

Fig. 5. Preparative runs for isolation of tryptic peptides. Peak analysis and assignments described in Table I and Fig. 6. Gradient, 0–45% B in 90 min on WP C_8 (A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile). Detection at 214 nm. About 300 μg equivalent of bSt injected.

dized and reduced material are best illustrated with the "large loop" fragment. A single peak for T5 plus T18 elutes at about 52 min in the oxidized preparation in Fig. 3 and two peaks, T5 and T18, elute at about 48 and 49 min, respectively, in the reduced preparation in Fig. 3. The small loop oxidized and reduced peptide fragments are more elusive. Two fragment peaks are tentatively identified in the oxidized preparation. One is T23 plus T25 as expected, and the other is T23 through T25 still containing the T24 arginine. Upon reduction the disulfide bond between T23 and T25 is cleaved. T23 elutes with the solvent front (as does the previously digested T24) while T25 has a longer retention and elutes near the T7 peak. The peptide backbone of the T23 through T25 fragment remains intact and the retention of this peak appears to be unchanged upon reduction. The peaks representing the small loop fragments elute in the window of 25–35 min along with T15 plus T16 and T2.

A consistent general order of elution as illustrated in Figs. 3–5 has been observed for the major peaks (height greater than 0.01 a.u.) including fragments T10, T3, T13, T14, T21, T15 plus T16, T2, T9, T18, T5 plus T18, T11 plus T12, T1 and T8. In the digests these are the most characteristic peaks of the peptide maps because they have the larger responses at UV 214 nm. The less intense fragments (height less

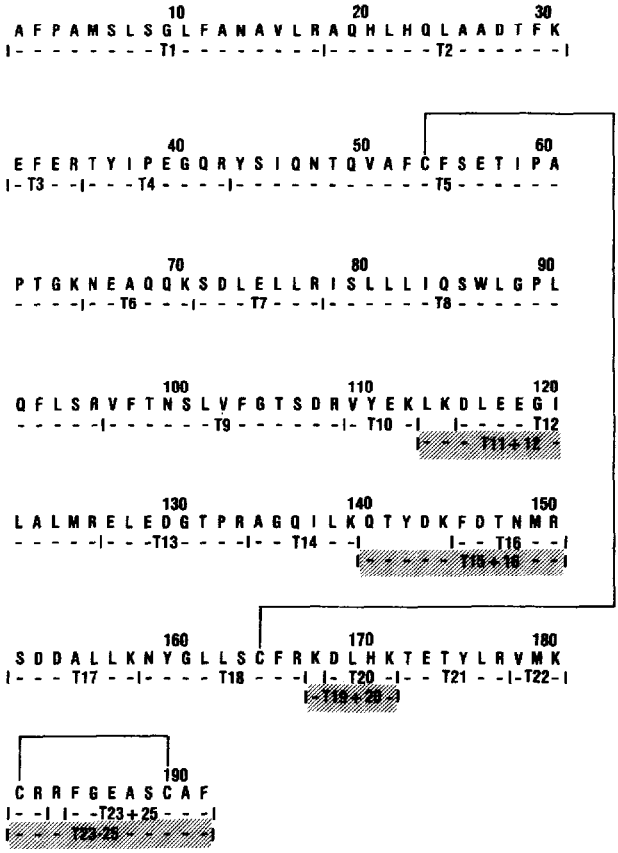

Fig. 6. Peptides observed in tryptic maps (see Fig. 5 and Table I). Note that the highlighted fragments have a bond which trypsin did *not* cleave.

than 0.01 a.u.) T6, T22, T20, T4, T7 and the small loop combinations were not always distinguishable from the background noise which was as high as 5% of full scale (0.005 a.u.). Additional work is required to assess the significance of these peaks and other small peaks which could be representative of bSt impurities.

The peptide mapping method can readily distinguish the differences between pituitary and recombinant derived bSt as illustrated in Fig. 3. The T1 peak is notably absent in the pSt maps. The T13 fragment contains the known heterogeneity (Val or Leu) at position 127 in the pituitary material. Extra peaks of reduced intensity relative to the rbSt maps are observed in this region (*ca.* 12 min in Fig. 3) of the pbSt pituitary peptide map. This heterogeneity and resulting extra peaks have not been observed in rbSt. Several extra peaks are also observed in pbSt maps which is consistent with additional heterogeneity in this material.

The combined fragments of T11 plus T12 and T15 plus T16 indicate that the digestion with trypsin does not go to completion at some sites which trypsin could cleave (see Fig. 6).

CONCLUSION

A peptide mapping procedure utilizing trypsin digestion RP-HPLC analysis and amino acid analysis has been developed for bovine somatotropin. This method is suitable for the qualitative comparison of the protein primary structure of samples and reference material.

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