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ARTICLES

Discrimination of Recombinant and Pituitary-Derived Bovine and Porcine Growth Hormones by Peptide Mass Mapping

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Somatotropins, which are used in cattle for growth and lactating performances, are difficult to reliably detect because no direct method exists. Reversed-phase high-performance liquid chromatography (RP-HLC) coupled to electrospray ionization quadrupole mass spectrometry (ESI/MS) has been developed to separate and characterize the N-terminal peptides resulting from tryptic cleavage of natural and recombinant growth hormones from different species (bovine, porcine, and human) and suppliers. Conditions for tryptic digestion were optimized. This technique was found to be optimal to cleave efficiently the N-terminal peptide of the proteins without releasing too much noise from the matrix. Characterization of the peptides through ESI(+)-MS allowed natural and recombinant growth hormones from bovine and porcine species with N-terminal amino acid sequences differing from one amino acid residue to be discriminated. However, the studied human growth hormones had similar primary sequences that did not permit any discrimination between recombinant and natural forms, thus confirming the known identity of these hormones. Protein digestions with pepsin and chymotrypsin were also compared but were not conclusive due to the too small N-terminal peptides released after proteolysis.

KEYWORDS: Growth hormones; somatotropins; human; bovine; porcine; tryptic mass mapping

INTRODUCTION

Growth hormones (GH), also known as somatotropins (ST), are proteins produced by the anterior pituitary gland. The biological effects of somatotropins are numerous and associated with growth, development, and reproductive functions (*1*−*4*). Because of their specific three-dimensional structure, which is essential for receptor binding and biological activity triggering, no interspecies biological activities have ever been reported (*5*). Growth hormones are widely used outside Europe to stimulate milk production in dairy cows and as a general growth promoter in pigs (*6*). Human growth hormone is thought to be widely abused in human sports because the increase in muscle size and strength they promote make them a viable alternative to anabolic steroids (*7*, *8*). Recombinant DNA techniques allow the production of large quantities of recombinant growth hormones, which may exhibit slightly different chemical structures from the pituitary somatotropin by the addition of a number of amino acids on the N-terminal side. Indeed, various forms of recombinant bovine growth hormones with slightly different N-terminal sequences have been produced and described in the literature (*9*). Recombinant bovine and porcine somatotropins from these sources, legally used in United States, are banned in the European Union; nevertheless, their illegal distribution and use is probable within the European Community (*10*, *11*). Thus, methods are required to discriminate natural somatotropins from recombinant ones.

Until now, the detection of growth hormone in bovine plasma, milk, and tissues has been based on radioimmunoassays and ELISA procedures (*12*−*15*); however, none of these methods allowed the endogenous and recombinant somatotropins to be distinguished. Some studies intended to discriminate between endogenous and recombinant bovine somatotropin standards have been reported (*11*, *16*), but no method is at present available because recombinant proteins from these sources have similar or very close sequences (addition of one to nine amino acids) compared to natural hormones, which makes their direct discrimination an analytical challenge. Successful attempts to detect directly recombinant hormone administration in animals have never been reported. Alternative methods have been developed, such as quantification through immunoassay techniques of secondary markers of ST administration such as insulin-like growth factor I (IGF-I): indeed, the main site of

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ST action is the liver, where they stimulate the production of IGFs (*15*, *17*, *18*).

Mass spectrometric peptide mapping has become an established and powerful structural tool for the analysis of proteins (*19*−*26*). This method is based on mass spectrometry analysis of the peptides released after specific enzymatic digestion of a protein. The analysis and characterization of the peptide mixture is performed by coupling HPLC directly with positive electrospray mass spectrometry [ESI(+)-MS]. Retention times and masses of different fragments depend on the amino acid sequence of each peptide and will then be modified by any amino acid residue addition, substitution, or deletion.

The aim of the present work was to develop an original method, based on somatotropin-specific proteolytic digestion followed by ESI-MS detection of the released peptides, that permits the discrimination between natural and recombinant growth hormones of different mammalian species (bovine, porcine, and human). Such an approach, never described until now, is planned to be the preliminary step in the detection of illegal administration of recombinant proteins.

MATERIALS AND METHODS

Materials. Pituitary-derived bovine somatotropins (bST) were obtained from ICN Biomedicals (Irvin, CA) and from the Harbor-UCLA Medical Center (Torrance, CA). Pituitary-derived human growth hormone (hST) was obtained from Sigma-Aldrich (St. Louis, MO) and from the Harbor-UCLA Medical Center. Pituitary-derived porcin somatotropins (pST) were obtained from both Sigma-Aldrich and the Harbor-UCLA Medical Center. Recombinant bovine somatotropins (rbST) were from Monsanto (St. Louis, MO) and Elanco (Lactotropin) (Indianapolis, IN). Recombinant human (rhST) hormones were obtained from ICN Biomedicals and Ares-Serono (St. Louis, MO), and porcine recombinant hormones (rpST) were from the Harbor-UCLA Medical Center. TPCK-treated porcine pancreas trypsin, chymotrypsin, and pepsin were purchased from Sigma-Aldrich. HPLC grade acetonitrile, methanol, dichloromethane, and acetic acid were from SDS (Peypin, France).

Methods. *Extraction of rbST from Lactotropin Syringes.* To extract rbST from the oily preparation contained in the syringe of Lactotropin, 15 mL of CH_2Cl_2 was added to 1.4 mL of syringe content, and the mixture was vortexed for 1 min. Twenty milliliters of deionized water was then added, and the mixture was homogenized and centrifuged (15 min, 15 °C, 300 rpm). The aqueous phase containing the recombinant bovine growth hormone was collected and freeze-dried.

Enzymatic Digestions. For tryptic and chymotryptic digestions, somatotropins were dissolved (1 mg/mL) in Tris−acetate buffer (100 mM, pH 8.0). Enzymes were dissolved in the same buffer at a concentration of 1 mg/mL and added to the substrates in a ratio of 1 to 20. The mixtures were vortexed for 1 min according to the Chang et al. (*23*) procedure. Hydrolysis were carried out for 24 h at 20 °C (unless other mentioned). Samples were stored at −18 °C until analysis. An identical procedure was followed for pepsic digestions, except that they occurred in citric acid solutions (100 mM, pH 2) instead of Tris−acetate buffer.

Kinetics of tryptic digestions were performed for 15, 20, 24, and 48 h at either 20or 37 °C.

HPLC-ESI/MS Measurements. A triple-quadrupole mass analyzer (Quattro LC, Micromass, Manchester, U.K.) was used, fitted with an electrospray ion source. The mass spectrometer was operated in the positive ion mode.

Somatotropin standards were prepared at 100 μg/mL in MeOH/H_2O (50:50, v/v), containing 0.1% acetic acid and introduced into the analyzer with a syringe pump set at 10 μL/min. The working conditions of the ion source and the mass spectrometer for standard solutions were as follows: capillary was set at 3 kV, cone voltage at 40 V, source temperature at 100 °C, drying gas temperature at 300 °C, acquisistion from *m*/*z* 500 to 2500 (time range, 6 s; interscan delay, 0.1 s; continuum mode). Nitrogen was used as nebulizer and drying gas at flow rates of 90 and 600 L/h, respectively. The data were collected and analyzed with the MassLynx software and the molar masses were calculated thanks to an algorithm that assigns charge states and transforms (deconvolutes) spectra to the zero charge domain.

Table 1. Theoretical and Calculated Molar Masses of Pituitary Bovine, Porcine, and Human Somatotropins (ST) and of Recombinant Bovine, Porcine, and Human ST from Different Suppliers

ST	supplier	theor mass (u)	calcd mass (u)
bST	Harbor-UCLA Medical Center	21815	21420/21810
	ICN Biomedicals	21815	21812
rbST	Monsanto	21875	21873
	Elanco	21875	21874
pST	Harbor-UCLA Medical Center	21727	21728
	Sigma Aldrich	21727	21730
rpST	Harbor-UCLA Medical Center	21798	21802
hST	Sigma Aldrich	22125	22125
	Ares-Serono	22125	22126
rhST	ICN Biomedicals	22125	22127
	Harbor-UCLA Medical Center	22125	22124

Fifty microliters of enzyme-digested ST (corresponding to 50 μg of hydrolyzed protein) was injected into an Alliance 2690 HPLC chromatograph (Waters, Milford, MA) equipped with a Vydac C_4 214TP54 column (5 μm, 300 Å, 4.6 × 250 mm) (Interchim, Montluçon, France). Peptides were separated using an elution gradient of CH_3CN/0.01% acetic acid (A) and H_2O/0.5% acetic acid (B), at a flow rate of 300 $\mu L \cdot min^{-1}$: the gradient started with 5% A increasing to 40% in 60 min, then to 60% in 5 min, then to 100% in 20 min, remaining at 100% A for 5 min, and finally decreasing to initial conditions (5% A) in 15 min.

Mass spectrometric analyses were done in the following working conditions: capillary was set at 3 kV, cone voltage at 45 V, acquisition from *m*/*z* 200 to 2000 (time scan, 4 s; interscan delay, 0.1 s; continuum mode), source temperature at 120 °C, and desolvatation temperature at 360 °C. Nitrogen was used as nebulization and desolvatation gas at flow rates of 90 and 600 L/h, respectively. The data were collected and analyzed with the MassLynx software.

RESULTS AND DISCUSSION

Undigested somatotropins generated about 10 multiply charged molecular ion peaks on the mass-to-charge (*m*/*z*) scale (**Figure 1**). Deconvolution of the multiple-charge states yielded the calculated masses as reported in **Table 1**.

The pituitary-derived bovine somatotropin contained two different proteins with masses of 21420 and 21810 u, the latter one being the most common variant of the protein, usually called bovine growth hormone. The difference between the masses of bST and rbST is due to the replacement of an Ala (71 u) with a Met (131 u) at the N-terminal end of the recombinant protein. The molecular mass of recombinant porcine somatotropin is different from the pituitary-derived one due to an additional Ala at the N-terminal end of the recombinant protein. No different masses were observed among the four studied human somatotropins, leading to the conclusion that their amino acid sequences were similar.

Direct LC-ESI/MS analysis of somatotropins can help in the discrimination of natural and recombinant hormones when their sequences are different; however, it implicates the use of a deconvolution program, which might emphasize the error on the calculated molecular masses, leading to ambiguous conclusions especially when very few amino acid residues are different. Direct discrimination of bovine growth hormones has also been reported by Borromeo and co-workers (*11*) on rbST contained in sustained-release forms; however, this

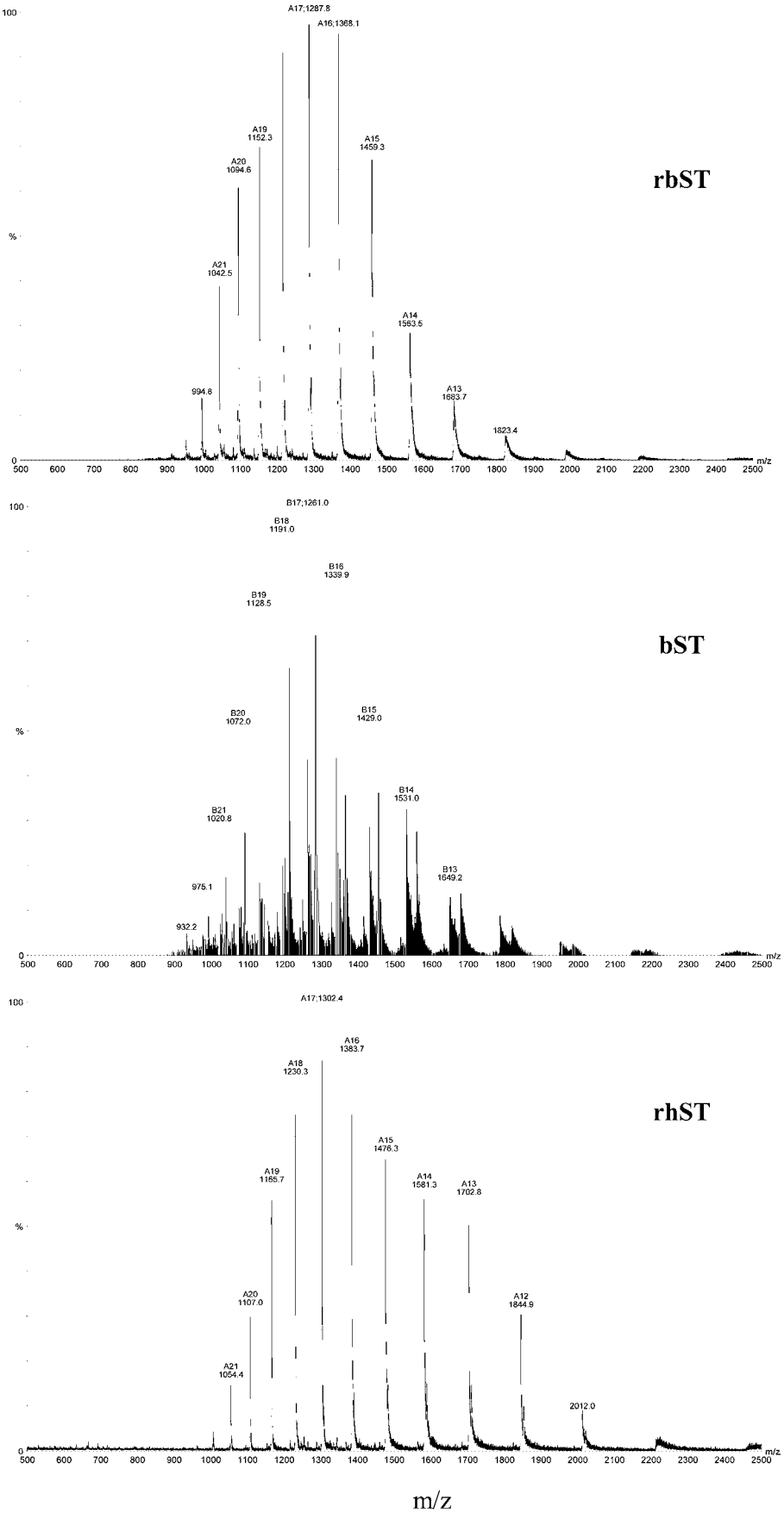

Figure 1. Examples of positive electrospray mass spectra of somatotropins. Mass spectra of intact recombinant bovine, pituitary bovine, and recombinant human growth hormones were obtained by flow injection analysis. Derived charges (*z*) of the multiply charged molecular ion peaks on the mass-to-charge scale are reported.

Table 2. Amino Acid Sequences[a] of the N-Terminal Fragments of bST, rbST, pST, rpST, hST, and rhST Based on Cleavage with Trypsin and Their Theoretical and Calculated Molecular Masses

ST	supplier	N-terminal sequence	theor mass (u)	calcd mass (u)
bST	Harbor-UCLA Medical Center	AFPAMSLSGLFANAVLR	1765	883/1765
	ICN Biomedicals	AFPAMSLSGLFANAVLR	1765	883/1765
rbST	Monsanto	MFPAMSLSGLFANAVLR	1825	913/1825
	Elanco	MFPAMSLSGLFANAVLR	1825	913/1825
pST	Harbor-UCLA Medical Center	FPAMPLSSLFANAVLR	1735	868/1735
	Sigma Aldrich	FPAMPLSSLFANAVLR	1735	868/1735
rpST	Harbor-UCLA Medical Center	AFPAMPLSSLFANAVLR	1806	903/1806
hST	Sigma Aldrich	FPTIPLSR	930	930
	Ares-Serono	FPTIPLSR	930	930
rhST	ICN Biomedicals	FPTIPLSR	930	930
	Harbor-UCLA Medical Center	FPTIPLSR	930	930

[a] Amino acid sequences of natural ST were obtained from literature data, whereas recombinant ones were deduced from calculations.

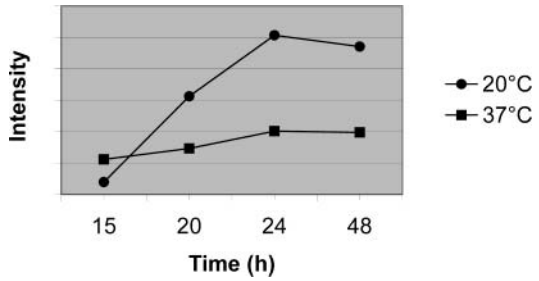

Figure 2. Kinetics of rbST digestion with trypsin. Kinetics was monitored over 48 h at 20 or 37 °C, and the amount of N-terminal peptide (*m/z* 1825), expressed as peak area intensities (LC-ESI/MS measurements, arbitrary units), was reported.

technique, based on MALDI-MS, might lead to the detection of aggregated molecules related to treatment and storage conditions.

The aim of the study was to develop a realistic methodology able to be used on a daily basis and dedicated to the unambiguous discrimination between pituitary ST and recombinant forms. The protein enzymatic digestion was evaluated in that sense, and the purpose was here to obtain significant N-terminal ends of each proteins according to enzyme cleavage specificity.

The sequences of the theoretical N-terminal fragments (fragments F1) based on specific cleavage of ST with trypsin on the C-side of Arg and Lys residues [except when followed by Pro (*27*)] and the theoretical molecular masses of the N-terminal peptides produced by digestion of the studied proteins are reported in **Table 2**. It appears that natural and recombinant bovine N-terminal fragments differ by one amino acid substitution, the number of residues remaining 17. Concerning porcine growth hormones, natural and recombinant proteins differ with the addition of an alanine residue at the N-terminal position. Human somatotropins (natural and recombinant) have the same N-terminal sequence consisting of eight amino acid residues.

To ensure that optimal conditions for N-terminal fragment release, we investigated the kinetics of rbST trypsic hydrolysis over 48 h at both 20 and 37 °C (pH and enzyme-to-substrate ratio being fixed parameters). Kinetics was monitored through the measurement of released quantities of rbST N-terminal end peptide (*m/z* 1825) (**Figure 2**). The N-terminal peptide is released as early as after 15 h of hydrolysis but in a very small amount at 20 °C. However, even if the digestion starts earlier at 37 °C (monitoring *m/z* 1825), it appeared that at 20 °C

Table 3. Amino Acid Sequences of the N-Terminal Fragments of bST, rbST, pST, rpST, hST, and rhST Based on Cleavage with Chymotrypsin or Pepsin and Their Theoretical and Calculated Molecular Masses

ST	supplier	N-terminal sequence	theor mass (u)	calcd mass (u)
bST	Harbor-UCLA Medical Center	AF	236	236
	ICN Biomedicals	AF	236	236
rbST	Monsanto	MF	296	296
	Elanco	MF	296	296
pST	Harbor-UCLA Medical Center	F	165	165
	Sigma Aldrich	F	165	165
rpST	Harbor-UCLA Medical Center	AF	236	236
hST	Sigma-Aldrich	F	165	165
rhST	ICN Biomedicals	F	165	165

hydrolysis allowed greater production of N-terminal end peptides. Conditions were finally set as follows: 24 h, 20 °C, pH 8, E/S 1/20. These optimized conditions differ slightly from the literature, where parameters such as 15 h/20 °C or 15 h/37 °C are often described for somatotropin trypsic hydrolysis (*23*, *26*).

The 11 hormones specimens were digested with trypsin, and the peptides were separated and characterized on an HPLC system coupled to a mass spectrometer through an electrospray ionization interface. To take into account only the peptides released from ST digestion, a correction for the enzyme blank was realized to eliminate peptides produced by trypsin self-digestion. **Figures 3**−**5** show the total ion current (TIC) chromatograms of the tryptic peptide mapping for different hormones. These chromatograms attest that the protein digestion has successfully occurred and that peptide fragments have been released.

Figures 3−**5** also report the chromatograms obtained by extracting the masses of F1 from the TIC chromatograms of each type of hormones. Bovine and porcine N-terminal peptides were detected as both singly charged species $[M + H]^+$ ions, [*m/z* 1765 (bST), *m/z* 1825 (rbST), *m/z* 1735 (pST), *m/z* 1806 (rpST)] and doubly charged ions $[M + 2H]^{2+}$ [*m/z* 883 (bST), *m/z* 913 (rbST), *m/z* 868 (pST), *m/z* 903 (rpST)], whereas human N-terminal peptides were detected only as singly charged species $[M + H]^+$ ions (*m/z* 930) (**Table 2**). **Figure 3** clearly shows different F1 signals for bST and rbST, with, respectively, ions at *m/z* 1825/913 and 1765/883 eluted at 78.6 and 77.6 min, respectively. rbST F1 eluted later than bST F1 because the Met

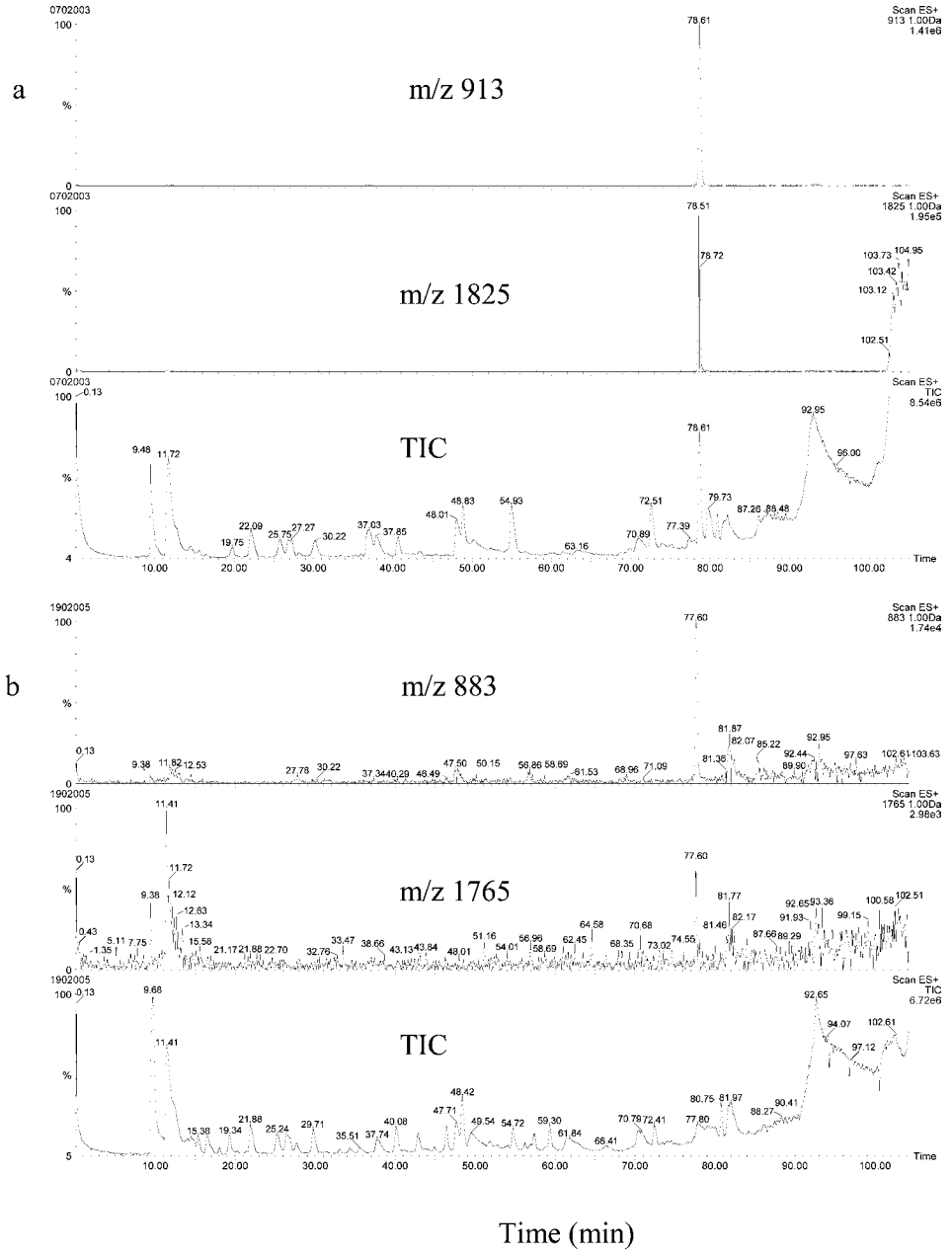

Figure 3. Ion chromatograms of bovine N-terminal tryptic peptides. HPLC-ESI ion chromatograms of tryptic peptides F1 are extracted from the TIC chromatograms of (**a**) rbST and (**b**) bST growth hormones after trypsin treatment.

residue in place of the Ala one gives the peptide a more hydrophobic behavior. **Figure 4** demonstrates in the same way that porcine F1 from either the recombinant or natural ST studied was different because ions occurring at *m*/*z* 1806/903 and 1735/868 were respectively identified at 77.3 and 77.7 min. The Ala residue gives recombinant F1 a more hydrophobic behavior; however, it also increases the length of the amino acid sequence, which can explain the very slight difference between the natural and recombinant F1 retention times. In **Figure 5**, the presence of an ion at *m*/*z* 930 in both natural and recombinant human somatotropin digests confirms that the N-terminal amino acid sequences from tryptic digestion of these two hormones are identical. From these examples, evidence is given that unambiguous discrimination of natural and recombinant standard somatotropins differing in one amino acid residue at their N-terminal ends can be achieved through trypsin hydrolysis. A previous tryptic mapping technique has already been successfully employed to identify the species origin of mammalian growth hormones (*20*, *23*, *24*, *26*); however, these studies were not led with the purpose of discriminating natural from recombinant species. For the first time, we report here a study aimed at discriminating, for a particular species, recombinant and natural hormones.

Somatotropin hydrolyses with other enzymes, pepsin and chymotrypsin, were also performed to evaluate the feasibility of other enzymatic peptide mappings to discriminate between natural and recombinant STs. Pepsin and chymotrypsin exhibit different mechanisms (pH, cleavage sites); however, in both cases, expected N-terminal ends were the same because both enzymes cleave on the C-side of Phe (**Table 3**).

Unlike trypsin, pepsin and chymotrypsin yielded small peptides for all studied hormones and, in particular, very small N-terminal peptides (one or two amino acid residues), which were not sufficiently N-terminal specific. Furthermore, in the case of digestion with pepsin, some of these N-terminal fragments (*m*/*z* 296 and 165, for example) were also generated

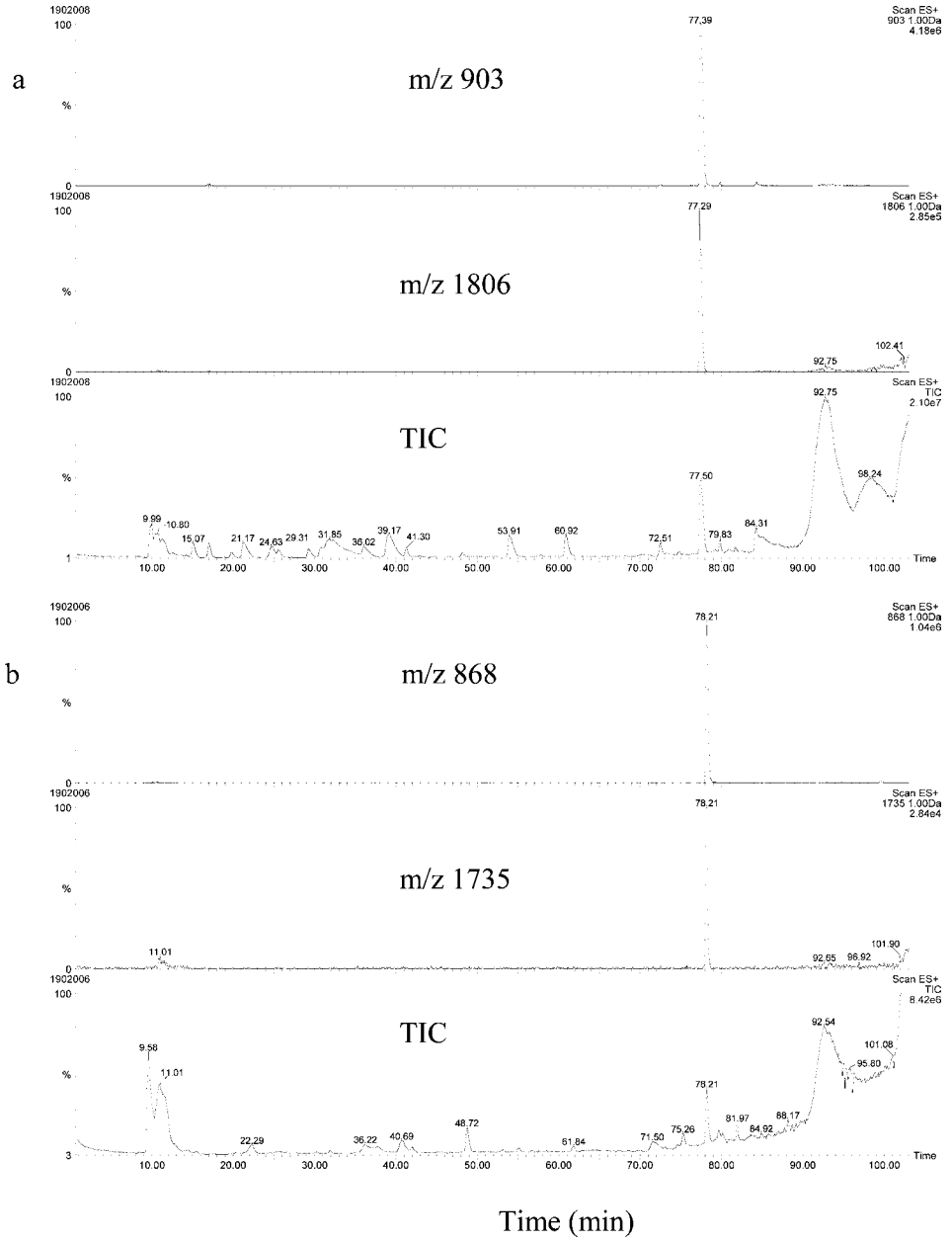

Figure 4. Ion chromatograms of porcine N-terminal tryptic peptides. HPLC-ESI ion chromatograms of tryptic peptides F1 are extracted from the TIC chromatograms of (**a**) rpST and (**b**) pST growth hormones after trypsin treatment.

by the enzyme alone (self-digestion), which makes it impossible to discriminate between natural and recombinant proteins. The attempt to use enzymes other than trypsin to obtain specific N-terminal fragments in order to discriminate natural and recombinant growth hormones was not decisive and may not be used unambiguously for that purpose.

In this paper, we have demonstrated a method for discrimination between endogenous and recombinant standards of bovine and porcine somatotropins. The study was based on injection on an LC-MS system of 50 μg of trypsin-digested protein, which corresponds to $\sim$5 μg of desired N-terminal peptide. The reproducibility of the overall method, an essential parameter for the reliability of the technique, was proven because five experiments were repeated for each hormone and the results averaged. This strategy applies to the screening of black market preparations in which somatotropin concentrations are high, as they are in Lactotropin syringes that have been successfully used in this study. Regarding the detection of illegal administration of hormones to animals, the screening of complex biological matrices such as plasma and milk would require the development of a specific method of extraction and purification, as well as improvement of our detection sensibility. Indeed, somatotropin concentrations, as calculated by RIA and ELISA, are 0.5 ng/mL in milk and 3 ng/mL in plasma of nontreated cows and 1 ng/mL in milk and 40 ng/mL in plasma of treated cows (*13*, *15*).

A strategy for the discrimination of bovine and porcine natural and recombinant commercially available growth hormones is reported. Despite the close homology between these hormones, the method described herein, based on tryptic mass mapping, allows the unambiguous identification of each somatotropin. Compared to indirect methods reported in the literature (RIA, ELISA), our technique allows, on the one hand, discriminattion between endogenous and recombinant forms and is, on the other hand, a direct and unambiguous evidence for the presence of recombinant hormones in a sample. This technique seems to be very promising and might be used to detect the presence of recombinant hormones in black market preparations as well as

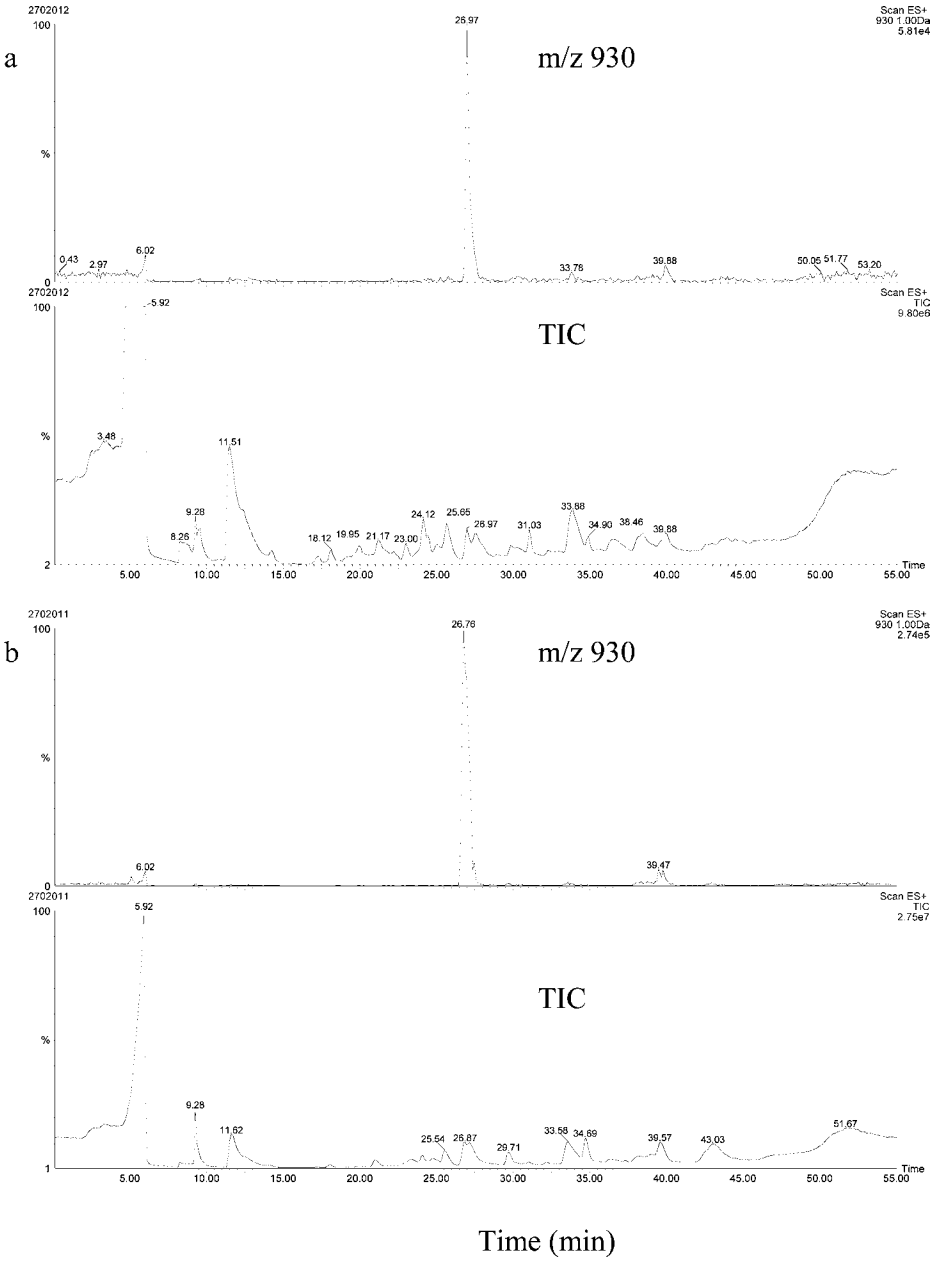

Figure 5. Ion chromatograms of human N-terminal tryptic peptides. HPLC-ESI ion chromatograms of tryptic peptides F1 are extracted from the TIC chromatograms of (**a**) rhST and (**b**) hST growth hormones after trypsin treatment.

in biological matrices of treated animals. In this paper, we have also demonstrated the limit of the peptide mass mapping strategy, which is not suitable when recombinant and endogenous STs exhibit the same primary structure. Different approaches, perhaps in the near future and based on an LC-C-IRMS system, should be useful in such cases.

Further work will focus on biological sample screening with the development of specific methods of extraction and purification of somatotropins from these complex matrices. Finally, to prove the feasibility and reliability of this strategy, validation of the method will have to be performed on animals after recombinant growth hormone administration and detection of the protein in milk or other matrices.

ABBREVIATIONS USED

bST, bovine somatotropin; DNA, deoxyribonucleic acid; ESI, electrospray ionization; E/S, enzyme-to-substrate ratio; GH, growth hormone; hST, human somatotropin; IRMS, isotopic ratio mass spectrometry; MS, mass spectrometry; pST, porcine somatotropin; rbST, recombinant bovine somatotropin; rhST, recombinant human somatotropin; rpST, recombinant porcine somatotropin; RP-HPLC, reversed-phase high-performance liquid chromatography; TIC, total ionic current.

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