Anal. Chem. **2008,** *80,* 4154–4160

Protein Quantification by Isotope Dilution Mass Spectrometry of Proteolytic Fragments: Cleavage Rate and Accuracy

Cristian G. Arsene,[†] Rüdiger Ohlendorf,[†] William Burkitt,[‡] Caroline Pritchard,[‡] André Henrion,*[,†] Gavin O'Connor,[‡] David M. Bunk,[§] and Bernd Güttler[†]

Department Metrology in Chemistry, Physikalisch-Technische Bundesanstalt, Braunschweig, D-38116, Germany, Department of Mass Spectrometry, LGC, Queens Road, Teddington, TW11 OLY, U.K., and Analytical Chemistry Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899-8392

The practice of quantifying proteins by peptide fragments from enzymatic proteolysis (digestion) was assessed regarding accuracy, reliability, and uncertainty of the results attainable. Purified recombinant growth hormone (rhGH, 22 kDa isoform) was used as a model analyte. Two tryptic peptides from hGH, T6 and T12, were chosen to determine the amount of the protein in the original sample. Reference solutions of T6 and T12 (isotopically labeled forms), value assigned by quantitative amino acid analysis (AAA) after complete hydrolysis, were used as internal standards. The accuracy of protein quantification by fragments T6 and T12 was evaluated by comparison of peptide results to those obtained for the same rhGH sample by AAA. The rate of cleavage (and thus the experimental protocol used) turned out to be crucial to the quality of results in protein quantification using enzymatic fragments. Applying a protocol customarily found in (qualitative) bottom-up proteomics gave results significantly higher than the target value from AAA (+11% with T6 and +6% with T12). In contrast, using a modified protocol optimized for fast and complete hydrolysis, results were unbiased within the limits of uncertainty, while the time needed for completion of proteolysis was considerably reduced (30 min as compared to 1080-1200 min). The method assessed highlighted three important criteria deemed necessary for successful protein quantification using proteolysis-based mass spectrometry methods. These are the following: the requirement for both the selected peptides and labeled internal standard to be stable throughout digestion; the correct purity assignment to the selected peptide standards; the proof of equimolar release of the selected peptides. The combined (overall) uncertainty for protein quantification was established by combination of estimates obtained for individual components and found to be $U = 4\%$ for this example. This uncertainty is of the same order as that typically attainable in quantification of "small" organic molecules using liquid chromatography/isotope dilution mass spectrometry.

The past decade has seen a significant increase in the development of mass spectrometric methods for protein quantification.[1,2] Although it is viable to directly analyze intact proteins[3,4] by liquid chromatography/mass spectrometry (LC/MS), most of the examples of quantification that have been described are based on specific cleavage by enzymatic proteolysis (digestion) of the proteins down to smaller fragments, most of which are still long enough in amino acid sequence to provide specificity for the precursor protein, even in a complex mixture.[5–14] This enables the simplification of the quantification process to the analysis of short sequences of amino acids which are amenable to standard LC/MS techniques. Using isotopically labeled forms of the peptides as internal standards potentially introduces the advantages of reliability, accuracy, and repeatability into protein quantification that have been demonstrated in the analysis of "small molecules" with isotope dilution mass spectrometry (IDMS).[15] Therefore, the measurement principle may be consid-

* To whom correspondence should be addressed. Phone: ++49 531592-3120. Fax: ++49 531592-3015. E-mail: andre.henrion@ptb.de.

[†] Physikalisch-Technische Bundesanstalt.

[‡] LGC Teddington.

[§] National Institute of Standards and Technology.

(1) Lill, J. *Mass Spectrom. Rev.* **2003**, *22*, 182–194.
(2) Righetti, P. G.; Campostrini, N.; Pascali, J.; Hamdan, M.; Astner, H. *Eur. J. Mass Spectrom.* **2004**, *10*, 335–348.
(3) Bunk, D. M.; Welch, M. J. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 1247–1254.
(4) Gordon, E. F.; Mansoori, B. A.; Carroll, C. F.; Muddiman, D. C. *J. Mass Spectrom.* **1999**, *34*, 1055–1062.
(5) Barr, J. R.; Maggio, V. L.; Patterson, D. G., Jr.; Cooper, G. R.; Henderson, L. O.; Turner, W. E.; Smith, S. J.; Hannon, W. H.; Needham, L. L.; Sampson, E. J. *Clin. Chem.* **1996**, *42*, 676–1682.
(6) Barnidge, D. R.; Dratz, E. A.; Martin, T.; Bonilla, L. E.; Moran, L. B.; Lindall, A. *Anal. Chem.* **2003**, *75*, 445–451.
(7) Gerber, S. A.; Rush, J.; Stemman, O.; Kirschner, M. W.; Gygi, S. P. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6940–6945.
(8) Barnidge, D. R.; Goodmanson, M. K.; Klee, G. G.; Muddiman, D. C. *J. Proteome Res.* **2004**, *3*, 644–652.
(9) Kuhn, E.; Wu, J.; Karl, J.; Liao, H.; Zolg, W.; Guid, B. *Proteomics* **2004**, *4*, 1175–1186.
(10) Havliš, J.; Shevchenko, A. *Anal. Chem.* **2004**, *76*, 3029–3036.
(11) Lu, Y.; Bottari, P.; Turecek, F.; Aebersold, R.; Gelb, M. H. *Anal. Chem.* **2004**, *76*, 4104–4111.
(12) Anderson, L.; Hunter, C. L. *Mol. Cell. Proteomics* **2006**, *5*, 573–588.
(13) Rivers, J.; Simpson, D. M.; Robertson, D. H. L.; Gaskell, S. J.; Beynon, R. J. *Mol. Cell. Proteomics* **2007**, *6*, 1416–1427.
(14) Keshishian, H.; Addona, T.; Burgess, M.; Kuhn, E.; Karr, S. A. *Mol. Cell. Proteomics* **2007**, *6*, 2212–2229.
(15) De Leenheer, A. P.; Thienpont, L. M. *Mass Spectrom. Rev.* **1992**, *11*, 249–307.

10.1021/ac7024738 CCC: $40.75
Published on Web 04/30/2008

ered as a basis for the development of reference measurement procedures for proteins at a quality level satisfying the needs in characterization of standard reference materials.[16] Systematic investigations into the precisions potentially attainable are required in this context, and the suitability of experimental protocols for obtaining accurate results is to be checked. Although these issues have been addressed,[5,8,13] properties such as the robustness of results toward changes in proteolysis conditions, which is crucial to comparability between laboratories (and, as a consequence, metrological traceability of results), have not been considered so far. In addition, no estimation of the lower limits of the measurement uncertainty to be expected with the technique has been evaluated. In practical applications, completeness of hydrolysis as necessary to avoid negatively biased results as well as value assignment of concentration to the reference solution of the labeled peptide fragment(s) used turn out to still be the factors limiting the accuracies obtained. So, for instance, biases in the range of 26−42% have been attributed to the combination of both sources of error, and the use of in vitro synthesized isotopically labeled proteins instead of tryptic peptides has been proposed as an alternative therefore.[17]

However, the present paper will demonstrate the applicability of protein quantification by IDMS of peptide fragments at a level of accuracy limited primarily by the properties of the LC/MS instrumentation used and discusses experimental requirements necessary for this to be achieved. The effects of experimental protocol on tryptic fragment generation and quantitative error were investigated. In addition to incompleteness of proteolysis, chemical modification at the amino acid side chains during the course of proteolysis was considered as a possible source of bias as it may compromise the results by changing the molecular mass of the fragments and consequently the amount virtually detected of them by mass spectrometry. To this end, the influence of the proteolysis rate on accuracy was investigated.

Human growth hormone, hGH (recombinant, 22 kDa isoform), was chosen as the model substrate. Though the technique is applicable to complex matrices, a solution of the purified recombinant protein (rhGH) in buffer was used as the test sample. This allows for an independent target value to be obtained on the solution by amino acid quantification after complete hydrolysis (amino acid analysis, AAA). The protease chosen was trypsin as this is used in the vast majority of proteomics applications. Two different methods for the enzymatic proteolysis of hGH were compared, a "slow" and a "rapid" one. Conditions applied to get a comparatively slow proteolysis were close to those in current practice for "bottom-up" protein identification by peptide mapping. In particular, proteolysis was performed in a purely aqueous buffer solution at a low concentration of trypsin, as the reaction was expected to proceed slowly in this case. This type of experiment will be referred to as *slow proteolysis protocol* here. The presence of organic solvents has been shown to possess the potential to significantly increase the rate of proteolysis with proteins that behave in a "protease-resistant" manner in aqueous medium.[18,19] The effect is attributed to reasons such as improved solubility and denaturing of the protein, while the activity of the enzymatic protease is often retained in water−organic solvent systems. Accelerated release of proteolysis fragments was therefore expected by pursuing a recently recommended method[19] using 80% acetonitrile−water as the digestion medium. This will be referred to as the *rapid proteolysis protocol*. Two fragments resulting from the cleavage of hGH by trypsin (T6 and T12) were selected for quantification of the protein according to the title method. The use of two fragments is to provide two independent measures of protein quantification, to add confidence to obtaining an unbiased result.

MATERIALS AND METHODS

Materials. Water was used as prepared in a MilliPore Milli-Q system (Bedford, MA) and of 0.055 μS cm^{-1} conductivity, at most. Acetonitrile used as solvent and as mobile phase was of CHROMASOLV grade by Sigma- Aldrich. Hydrochloric acid was "constant boiling (6N)" (Pierce, Rockford, IL). $CaCl_2$ was Calciumchlorid- Tetrahydrat, Suprapur from E. Merck, Darmstadt, Germany. TRISMA hydrochloride, TRIS-base, acetic acid (Fluka, BioChemika Ultra, for luminescence, ≥99.5%), iodoacetamide (alkylating reagent), dithiothreitol (DTT, DL-dithiothreitol, for electrophoresis, 99%), and trypsin (proteomics grade, 20 μg/ampule) were from Sigma-Aldrich. RapiGest- SF (1 mg/ampule) had been obtained from Waters Corp. (Milford, MA). Isoleucine (≥99.5%), leucine (≥99.5%), phenylalanine (≥99.0%), proline (≥99.5%), valine (≥99.5%), and threonine (≥99.5%), all L-isomers and isotopically natural forms, were BioChemika Ultra grade from Fluka (Buchs, Switzerland). Purities are given in parentheses. The isotopically labeled amino acids (L-isoleucine-$^{13}C_6$, L-leucine-$^{13}C_6$, L-phenylalanine-$^{13}C_9$,^{15}N, L-proline-$^{13}C_5$, L-valine $^{13}C_5$,^{15}N, and L-threonine-$^{13}C_4$,^{15}N) were all 98% in isotopic enrichment and were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). [Note: Certain commercial equipment, instruments, or materials are identified in this paper to specify experimental procedures adequately. Such identification is not intended to imply recommendation or endorsement by Physikalisch-Technische Bundesanstalt, LGC, or the National Institute of Standards and Technology, nor is it intended to imply that materials or equipment identified are necessarily the best available for the purpose.]

Reagent Solutions. Tris(hydroxymethyl)aminomethane buffer solution (TRIS) was made by dissolving TRISMA hydrochloride and TRIS-base in equimolar ratio in water. Trypsin solution, 45 and 90 ng/μL, were obtained by dissolving the content of 1 ampule (20 μg) of trypsin and 2 ampules, respectively, in 443 μL of acetic acid (50 mmol/L). Trypsin solutions were activated by equilibration to 37 °C prior to addition to the reaction tubes. DTT solutions were prepared by dissolving dithiothreitol in either TRIS or water as needed. Hydrochloric acid (HCl) was prepared by dilution from the "constant boiling" 6 N stock solution. Solutions of amino acids (natural as well as isotopically labeled) used with amino acid quantification were prepared in water.

Sample Solution of Human Growth Hormone. Recombinant human growth hormone (hGH; 22 kDa isoform) was purchased from ProSpec-Tany TechnoGene (Rehovot, Israel). A 10 mg amount of the lyophilized material was further purified by

(16) Tai, S.S.-C.; Welch, M. J. *Anal. Chem.* **2005**, *77*, 6359–6363.
(17) Brun, V.; Dupuis, A.; Adrait, A.; Marcellin, M. Thomas, D.; Court, M.; Vandenesch, F.; Garin, J. *Mol. Cell. Proteomics*, **2007**, *6*, 2139–2149.
(18) Russell, W. K.; Park, Z. Y.; Russell, D. H. *Anal. Chem.* **2001**, *73*, 2682–2685.
(19) Strader, M. B.; Tabb, D. L.; Hervey, W. J.; Pan, C.; Hurst, G. B. *Anal. Chem.* **2006**, *78*, 125–134.

liquid chromatography on a RP-18 column (Phenomenex, Jupiter, 300 Å, 250 mm × 10 mm) using a mobile phase of acetonitrile/trifluoroacetic acid (0.1%) in water 56:44 (v/v) in isocratic mode at a flow rate of 1.5 mL/min. The collected fraction was freeze-dried and redissolved in 9 mL of 50:50 acetonitrile/water (v/v).

Solutions of hGH Cleavage Fragments T6 and T12 in Isotopically Labeled Form. T6* and T12*, ~2 mg of each, were obtained from Thermo Electron GmbH (Ulm, Germany). T6* (YSFLQNPQTSL* CFSESIPTPSNR) was isotopically labeled by placing leucine-U-$^{13}C_6$ (98.0%),$^{15}N_1$ (98.0%) in position 11, while proline-U-$^{13}C_5$ (99.5%),$^{15}N_1$ (98.3%) was used in position 6 with T12* (LEDGSP* R). The T6* material (0.8 mg) was further purified by liquid chromatography as described above with the hGH, except that the fraction of acetonitrile was reduced here to 30% with the mobile phase. The reference solution of T6* was obtained by redissolving the resulting product in 2 mL of water. T12* was used without further purification. The reference solution of T12* was prepared by dissolving 1.5 mg of the freeze-dried material in 2 mL of water. Concentrations were determined by quantitative amino acid analysis (see corresponding subsection) and found to be 102.55 nmol/g with the T6* solution and 146.81 nmol/g with T12*.

Determination of Amino Acid Containing Impurities in the T6* and T12* Reference Solutions and in the hGH Sample Solution. Both reference solutions as well as the hGH sample solution were checked for peptide impurities (i.e., peptides other than T6* and T12*) which may be formed as unwanted byproducts in the syntheses. To this, mass spectra measured with mass ranges of m/z 50 to m/z 3000 were evaluated on direct infusion of the solutions using a time-of-flight instrument. Several peptides were identified by their exact masses in the T6* solution which contained T6* plus or minus an extra amino acid (+S, +T, +L/I, +E, −L/I, −T), whereas just one was found in the T12* solution, corresponding to T12*+S. The amounts of these impurities relative to T6* and T12* were quantified by reversed-phase LC/ESI-quadrupole MS in selected ion mode monitoring m/z 1312.7 (T6*), 1334.6 (T6*+S), 1338.1 (T6*+T), 1341.1 (T6*+L/I), 1345.0 (T6*+E), 1284.5 (T6*−L/I), 1287.4 (T6*−T), 390.4 (T12*), and 434.0 (T12*+S). Assuming the response factors of the impurities being close to those of T6* and T12*, the chromatographic peak areas were used to calculate the fractions of peptide admixtures in T6* and T12*. These were 6.8% with T6* and 1.6% with T12* (mean values of $n = 3$ runs). No amino acid containing impurities had been detected in the hGH solution after purification. Correction factors derived from this to be applied to the results of quantification by AAA were 0.932 (T6*), 0.984 (T12*), and 1.00 (hGH).

Determination of hGH, T6, and T12 by Quantitative Amino Acid Analysis. Hydrolysis was done by placing the freeze-dried residue of 50−150 μL aliquots of the pertaining solution at the bottom of a 5 mL vacuum hydrolysis tube (Pierce, Rockford, IL), adding 400 μL of 6 mol/L HCl, evacuating, and keeping the tube at 150 °C for 60 h. The amino acids released were quantified by isotope dilution mass spectrometry using the "double exact matching" method, the details of which are given elsewhere.[20–22] To this end, isotopically labeled amino acids were added in defined amounts to the aliquots prior to hydrolysis. Calibration was based on reference solutions of the pure amino acids that were treated in the same way as the sample aliquots. In particular, the same amount of isotopically labeled amino acids was added to both the reference solution and the corresponding sample solutions. Using this method, any bias that might be caused by incomplete isotopic enrichment inherent in the labeled amino acids would be corrected for. An LC/ESI-quadrupole MS instrument was used for measurements. The amino acids were quantified using ion traces at m/z116.1/121.1 (proline), m/z 118.1/124.1 (valine), m/z 120.1/125.1 (threonine), m/z 132.1/138.1 (leucine and isoleucine), and m/z 166.1/176.1 (phenylalanine). The primary results were corrected by the factors obtained in determination of amino acid containing impurities as described above.

Proteolysis According to the Slow Protocol. Aliquots of 21 mg of T6* reference solution and 14.7 mg of T12* reference solution were added to 70 mg of the hGH reference solution. The mixture was lyophilized prior to adding 200 μL of 100 mmol/L TRIS buffer containing 5 mmol/L DTT and 2 g/L RapiGest. After incubation (10 min at 100 °C and then 60 min at 40 °C), 10 μL were added of a 0.3 mol/L solution of iodoacetamide in 100 mmol/L TRIS for alkylation. After leaving the mixture for 1 h in the dark, excess of iodoacetamide was quenched by addition of DTT in 3-fold excess (30 μL of a 0.3 mol/L solution of DTT in 100 mmol/L TRIS). Then, another 100 μL of TRIS buffer was added, in order to reduce the RapiGest concentration to 1.2 g/L. Proteolysis was started by spiking with 20 μL of activated trypsin solution (90 ng/μL) and bringing the mixture to 37 °C. Samples of 20 μL were drawn after 10, 20, 40, 80, 160, 1080, 1440, 2520, 2880, 3960, and 8640 min. Proteolysis was stopped with the samples by addition of 20 μL of HCl (150 mmol/L). After each sample was withdrawn, a fresh aliquot of activated trypsin (20 μL) was spiked into the reaction mixture. Relative amounts of T6, T6*, T12, and T12* were monitored by reversed-phase LC/ESI-quadrupole MS using ion traces at m/z 892.3 (T6, $z = 3$), m/z 894.6 (T6*, $z = 3$), m/z 387.4 (T12, $z = 2$), and m/z 390.4 (T12*, $z = 2$).

Proteolysis Using the Rapid Protocol. A 340 μL aliquot of a buffer containing TRIS at 50 mmol/L and $CaCl_2$ at 10 mmol/L in 80% acetonitrile was added after lyophilization of a mixture of 21 mg of T6* reference solution, 14.7 mg of T12* reference solution, and 70 mg of the hGH reference solution. Proteolysis was started by addition of 20 μL of activated trypsin (90 ng/μL) and the mixture kept at 37 °C as with the slow proteolysis. Samples of 20 μL each were taken after 10, 20, 40, 80, 160, 1080, 1440, 2520, 2880, 3960, and 8640 min. A 80 μL aliquot of DTT (6.25 mmol/L) was added to each of the samples, which were left at room temperature for 1 h followed by lyophilization and dissolving the residue in 40 μL of HCl (37.5 mmol/L). After each sample draft a fresh aliquot of activated trypsin (20 μL) was added to the reaction mixture. Relative amounts of T6, T6*, T12, and T12* were monitored by reversed-phase LC/ESI-quadrupole MS using ion traces at m/z 873.3 (T6, $z = 3$), m/z 875.6 (T6*, $z = 3$), m/z 387.4 (T12, $z = 2$), and m/z 390.4 (T12*, $z = 2$).

Liquid Chromatography/Mass Spectrometry. Purity checks of the T6*, T12*, and hGH solutions were done on a micrOTOF-Q (Bruker Daltonik GmbH, Bremen, Germany) in direct infusion

(20) Henrion, A. *Fresenius J. Anal. Chem.* **1994**, *350*, 657–658.
(21) Milton, M. J. T.; Wielgosz, R. I. *Metrologia* **2000**, *37*, 199–206.
(22) Burkitt, W. I.; Arsene, C.; Henrion, A.; Bunk, D.; O'Connor, G. *Anal. Biochem.* **2008**, *376*, 242–251.

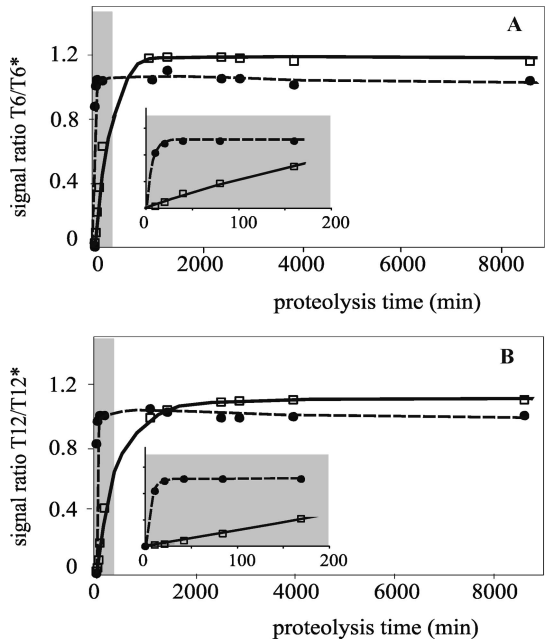

Figure 1. Formation of fragments T6 (A) and T12 (B) from hGH under conditions of slow proteolysis (open squares, solid line) and rapid proteolysis (filled circles, dashed line). Intensities of T6 and T12 are plotted relative to isotopically labeled internal standards T6* and T12*.

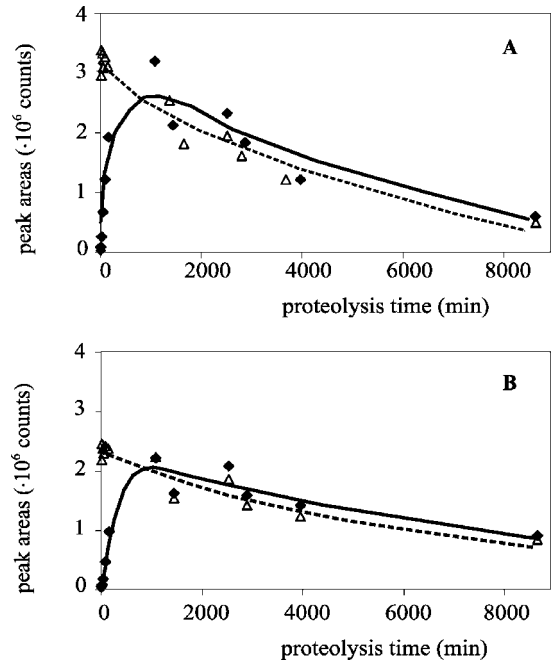

Figure 2. Timed profiles of individual signals: (A) T6 (filled diamonds, solid line) and labeled internal standard T6* (triangles, dashed line); (B) T12 (filled diamonds, solid line) and labeled internal standard T12* (triangles, dashed line).

ESI (positive ion) mode infusing the pertaining solution at 3 μL/min. Quantification of peptide impurities as well as AAA and monitoring of T6/T6* and T12/T12* ratios in the proteolysis experiments were performed on a Hewlett-Packard Series 1100 LC/MSD.

A Jupiter C18 300 Å column (5 μm, 150 mm × 2.0 mm, Phenomenex) was used in quantification of peptide impurities and in monitoring of T6/T6* and T12/T12* ratios. The mobile phase was acetonitrile (containing 1 mL/L formic acid)−water (containing 1 mL/L formic acid) with 0% acetonitrile at $t = 0-3$ min followed by a linear gradient bringing the acetonitrile content up to 80% within 27 min. The flow rate was 0.2 mL/min. Separation of amino acids in AAA was done on a ZIC-HILIC analytical column (3.5 μm, 150 mm × 2.1 mm, SeQuant GmbH, Haltern am See) using acetonitrile−water as mobile phase at 80:20 (v/v) containing 5 mmol/L ammonium acetate at 0.1 mL/min flow rate.

RESULTS AND DISCUSSION

Protocol and Product Formation. Time courses for proteolysis product formation from hGH with both types of protocol, slow and rapid, are shown in Figure 1. The initial concentration of trypsin was 5.3 mg/L in both cases, while the hGH concentration was at 132 mg/L (5.9 μmol/L), which makes a protease to protein ratio of 1:25 (w/w). Intensities of mass spectrometric signals of the cleavage products T6 and T12 are plotted relative to those of T6* and T12* (isotopically labeled variants of T6 and T12 which had been added as internal standards). Technically, the point of no further change in signal ratio may be considered to be the end point of proteolysis, where the digestion is complete with regard to the formation of measured tryptic peptides. For this to be reached with the slow protocol, it takes at least 1080−1200 min (18−20 h), while it is less than 60 min (1 h) using the rapid protocol.

Evaluation of timed profiles of the individual signals (Figure 2) reveals that once the fragments have been released from the protein their signals are falling off due to degradation or chemical modification. The difference in signal ratios obtained with slow and rapid digestion (Figure 1) can be accounted for by the assumption of degradation/modification not to take place on the fragments (T6 and T12) prior to their release from the intact protein sequence, or at least significantly slower there. Extinction of the signals would be delayed in that case with the fragments formed by cleavage from the protein compared to the isotopically labeled standards (T6* and T12*) which are present from the start. As a consequence, the signal ratios T6/T6* and T12/T12* as observed are expected not to reflect the correct amount-of-substance ratios hGH/T6* and hGH/T12* but to be positively biased under conditions of a slow evolution of the cleavage products. Indeed, the ratios found after complete hydrolysis are elevated on slow digestion if compared to rapid reaction conditions with either of the fragments. The effect is more prominent in the case of T6 than in T12, which is in line with the higher rate of decay observed with T6 (Figure 2).

Although the aim was not to fully determine the modifications occurring, two possible degradation pathways—nonspecific cleavages and deamidation—were considered. Stability tests were performed on the labeled peptides in both the presence and absence of trypsin, and peptide degradation was still observed (data not shown), suggesting the loss of signal over time is not due to nonspecific enzymatic cleavages. Another possible modification of peptides during proteolysis is deamidation of Asn and Gln. Deamidated peptides are shifted in mass by +0.984 *u*; this mass shift was observed by LC/MS for T6 (Figure 3).

Growth hormone is known for deamidation at sites Asn149, Asn152, and Gln137; however, in the region of T6, even after alkaline treatment at pH 8.5 and proteolysis, no deamidation has

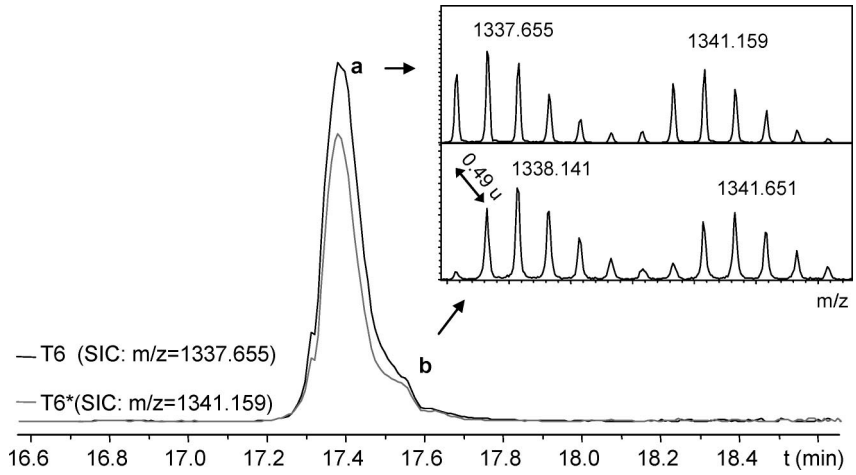

Figure 3. Result of analysis of the sample solution after 32 h proteolysis time (slow digest). The selected ion chromatograms (SIC) of both T6 and T6* are displaying a shoulder (b) next to the main signal (a) attributable to deamidated T6 and T6*. A corresponding mass shift (0.49 u at charge state $z = 2$) is found in the mass spectra (inset) taken at the respective retention times.

Table 1. Results of Direct Determination of hGH Content by Amino Acid Quantification

	amount-of-substance content, nmol/g					
run	Ile	Leu	Phe	Pro	Val	Thr
1	242.20	798.20	402.69	241.73	216.73	312.37
2	245.94	801.78	399.90	243.99	221.60	315.40
3	244.95	800.26	394.52	245.72	215.71	306.67
mean	244.36	800.08	399.04	243.81	218.01	311.48
CV %	0.79	0.22	1.04	0.82	1.44	1.42
ν^a	8	26	13	8	7	10
hGH mean	30.55	30.77	30.70	30.48	31.14	31.15
hGH mean	30.80					
CV %	0.93					

[a] "Stoichiometric factor" (times of occurrence of that amino acid in the 22 kDa hGH molecule).

Table 2. Results of hGH Determination by Quantification of the Tryptic Cleavage Products T6 and T12

	amount-of-substance content, nmol/g	
run	T6	T12
(a) Slow Digestion (Aqueous Conditions)		
1	34.11	32.79
2	34.49	33.17
3	33.88	32.44
4	34.36	32.79
mean	34.21	32.80
CV %	0.79	0.91
bias %[a]	11.1	6.5
(b) Rapid Digestion (80% Acetonitrile)		
1	30.90	31.06
2	30.24	31.03
3	30.48	30.74
4	29.72	30.91
5	30.03	31.01
6	30.00	30.96
mean	30.23	30.95
CV %	1.38	0.38
bias %[a]	−1.85	0.49

[a] Difference to the value obtained by amino acid quantification (see Table 1).

been described thus far.[23,24] In the present experiment the T6* appears to undergo deamidation while the corresponding segment in the protein was not observed to be modified at the beginning of proteolysis. This modification is a possible contribution to the distortion of the signal ratio T6/T6*. However, there must also be further contributions, as yet uninvestigated, relating to why the signal ratio of T12, which contains no potential deamidation sites, is biased too.

Quantification Result by AAA. Results of hGH determination by AAA for the sample solution are compiled in Table 1. Isoleucine (Ile), leucine (Leu), phenylalanine (Phe), proline (Pro), valine (Val), and threonine (Thr) released from the protein by acid hydrolysis had been quantified using liquid chromatography/isotope dilution mass spectrometry (LC/IDMS). Results of three replicate analyses had measurement uncertainties (CVs) of less than 1.5% for all of the amino acids. The figures obtained by calculating the hGH content on the basis of each of the individual amino acids result in a mean of 30.8 nmol/g and a CV of approximately 1%. The most significant potential sources of the overall uncertainty (inaccuracy) associated with AAA are (i) the presence of impurities (peptides/proteins) producing amino acids in addition to the analyte and (ii) incomplete hydrolysis. The first was reduced by purification of the hGH material prior to preparation of the substrate solution and subsequent checking of the solution for remaining impurities by direct infusion/time-of-flight (TOF) mass spectrometry (spectrum not shown). Completeness of hydrolysis under the experimental conditions used had been tested by a series of preliminary experiments confirming that on hydrolysis times longer than those used (60 h), the amount of amino acids formed did not increase (details not shown here). Additionally, the finding that the results in Table 1 do not scatter more "between amino acids" than "within amino acids" is an indirect support of both of the assumptions.

Results by Quantification of Proteolysis Fragments. Quantification results obtained using T6 and T12 as "substitute"

(23) Lewis, U. J.; Singh, R. N.; Bonewald, L. F.; Seavey, B. K. *J. Biol. Chem.* **1981**, *256*, 11645–11650.
(24) Hepner, F.; Cszasar, E.; Roitinger, E.; Lubec, G. *Proteome Sci.* **2005**, *3*, 1.

measurands are listed in Table 2. In this case, the amounts of T6 and T12 generated by enzymatic cleavage of the protein were measured by LC/IDMS. T6* and T12* were added in defined amounts to the protein solution prior to sample treatment and results calculated in the common way by combining the signal ratios observed for T6/T6* and T12/T12* with the known concentrations of T6* and T12*. The repeatability (CV) of the analysis procedure is better than 2%, independent of which of the proteolysis strategies is pursued (Table 2). In this example, using standard statistics (Bartlett's test[25]) the assumption of homogeneity is maintained at the 5% level risk of error and a pooled estimate can be derived for the type of method of CV = 0.97%. This is similar to what can typically be achieved in LC/IDMS of "small molecules", if working at a level well above the limit of detection. Still, the data are indicative of risks of uncertainties associated with the way in which proteolysis is being done. In the present case, if proteolysis is done under conditions of the slow protocol, results are obtained that are significantly biased with respect to the target value determined by AAA. Moreover, the extent of this is dependent on which of the cleavage products is being evaluated (bias, +11% with T6 and +6% with T12). In contrast to this, results agree with expectation if using the approach of rapid proteolysis in 80% acetonitrile. This outcome is consistent with the assumption that chemical modification of the cleavage products, as is inevitable with longer proteolysis times, imposes a systematic error on the quantification results.

Uncertainties of Results. Estimates of overall uncertainties were set up according to pertaining guidelines[26] by combining the contributions of the identified individual sources. With AAA, as used here, these are (i) uncertainty of the mass fractions (purities) of amino acids used in the preparation of the reference solutions, (ii) inaccuracy resulting from incompleteness of hydrolysis and/or admixtures (impurities) of the hGH, and (iii) a random component describing the distribution of results on repeating the measurement under the conditions given (repeatability). Considering the "worst" purity among the data provided by the manufacturer (phenylalanine, ≥99%) an interval of $a = 0.01$ (1%) half-width was assumed to appropriately describe this uncertainty component. The risk of inaccuracy had been minimized by measures described earlier to ensure completeness of hydrolysis and ascertain purity of hGH. Any remaining effect of this kind is expected to be reflected in a discrepancy of results between amino acids. Therefore, the coefficient of variation CV = 0.93% (see Table 1) was used as the measure for this uncertainty contribution. The repeatability component was estimated by the standard deviation of the mean of results, $s_{mean} = CV/\sqrt{6} = 0.38\%$. From this, a combined standard uncertainty was calculated of $u_c = 0.36$ (1.2%) and an expanded uncertainty of $U = 0.71$ (2.3%), which corresponds to a 95% confidence interval for the result (30.8 nmol/g).

Applying the same reasoning, the pooled standard deviation of results of quantification by fragments, CV = 0.97%; see Table 2 was used as the component accounting for potential remaining incompleteness of proteolysis and $s_{mean} = CV/\sqrt{6} = 0.40\%$ for repeatability. Instead of considering the mass fractions of the amino acids used, this time it is necessary to estimate the uncertainty associated with determination of the amount-of-substance content of the T6* and T12* in the reference solutions from which the internal standards had been added to the hGH samples. These were obtained by AAA using the same experimental setup and protocol as with hGH quantification by AAA. Therefore, the uncertainty estimate obtained for the hGH result by AAA, $u_c = 0.36$ (1.2%), were deemed applicable to the results for T6* and T12* concentrations in the reference solutions too. Another contribution to the overall uncertainty results from a potential difference in the fraction of peptides (natural vs labeled) covered under the mass spectrometric conditions applied. Using the data provided for isotopic purities of the labeled amino acids (see Materials and Methods), the resulting isotopic patterns of the peptides were calculated. The fractions obtained were 88.5 (T6*) and 90.1% (T6) as well as 90.5 (T12*) and 91.7% (T12). From this, the upper limit of the uncertainty contribution caused by mismatch of isotopic patterns was derived to be 2.0% (or less).

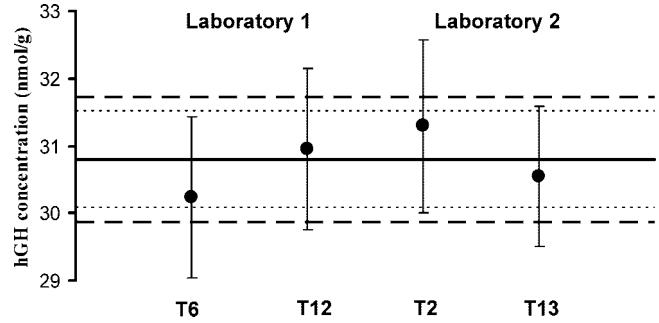

Figure 4. Results for the sample solution of hGH quantification with different proteolytic fragments T2, T6, T12, and T13. Bold line: Concentration of the hGH sample solution (mean result), determined by amino acid analysis. Dotted lines: Expanded relative uncertainty ($\pm U$) of the mean. Dashed lines: ±3% interval round the mean. Data points and error bars: Results obtained by quantification of the fragments and expanded uncertainties ($\pm U$) corresponding to 95% confidence intervals.

Combined, the individual sources of uncertainty sum to $u_c = 0.60$ (2.0%) and $U = 1.20$ (4.0%), which apply as estimates to hGH quantification using either T6 and T12. A graphical representation of results and confidence intervals (expanded uncertainties) is given in Figure 4. A drawback inherent to the method of estimating the overall uncertainty by combining individual components is that contributions which are not recognized are not considered and therefore result in underestimation. In order to check for the presence of hidden components, the data were compared to results obtained independently on the same hGH solution by another laboratory,[27] which used a similar approach but different fragments for quantification (T2 and T13). Agreement between both laboratories was within 2%. This evidence supports both the accuracy of the figures obtained using the rapid digest (80% acetonitrile) as well as confirming the completeness of the uncertainty budget.

(25) Anderson, R. L.; Bancroft, T. A. *Statistical Theory in Research*; McGraw-Hill: New York, 1952.

(26) *Guide to the Expression of Uncertainty in Measurement*; ISO: Geneva, Switzerland, 1993.

(27) Pritchard, C.; O'Connor, G. Personal communication.

CONCLUSION

Completeness of enzymatic cleavage is a necessary condition for accurate results in the absolute quantification of proteins using proteolysis fragments but is not sufficient in and of itself. In addition, experimental protocols have to be optimized toward high proteolysis rates so as to avoid bias resulting from, e.g., peptide modification proceeding faster on the internal standard than on the same stretch of sequence within the intact protein, released as a fragment. Supplementary to applying high concentrations of both substrate−protein and protease, high percentage organic/aqueous solvent mixtures may be useful, as has been demonstrated here with the system hGH/trypsin/acetonitrile (80%). Following these precautions, absolute quantification of proteins by IDMS of proteolytic fragments is eminently possible at an accuracy level of 4%, close to what is seen with analysis of small organic molecules using LC/IDMS. However, it is incumbent on the practitioner to prove these criteria have been satisfied before confidence can be assumed in proteomic type quantification.

ACKNOWLEDGMENT

The authors thank Dr. Klingel (Thermo Electron, Ulm, Germany) for kindly providing T6* and T12* peptides, used in this study.

Received for review December 4, 2007. Accepted March 10, 2008.

AC7024738