Mass Spectrometric Detection of Attomole Amounts of the Prion Protein by nanoLC/MS/MS

Bruce Onisko,[a] Irina Dynin,[a] Jesús R. Requena,[b] Christopher J. Silva,[a] Melissa Erickson,[a] and John Mark Carter[a]

[a] Western Regional Research Center, United States Department of Agriculture, Albany, California, USA
[b] Prion Research Unit, Department of Medicine, School of Medicine, University of Santiago, Santiago, Spain

Sensitive quantitation of prions in biological samples is an extremely important and challenging analytical problem. Prions are the cause of several fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs). At this time, there are no methods to diagnose TSEs in live animals or to assure a prion-free blood supply for humans. Prions have been shown to be present in blood by transfusion experiments, but based on the amount of infectivity found in these types of experiments, the amount of misfolded prion protein in blood is estimated to be only 30 to 625 amol/mL. More sensitive detection of prions in brain would allow earlier detection of disease and assure a safer food supply. We studied quantitation of the prion protein by use of nanoscale liquid chromatography coupled to a tandem mass spectrometer using the multiple reaction monitoring mode of operation. We developed a method based on the detection of VVEQMCTTQYQK obtained by reduction, alkylation, and digestion with trypsin of the prion protein. Detection of VVEQMCTTQYQK was more sensitive than for the derivative with phenylisothiocyanate (PITC) because of decreased ionization efficiency of the PITC-derivatized peptides. The VVEQMCTTQYQK method has a LOD of 20 to 30 amol for pure standards. Proof of principle is demonstrated by quantitation of the amount of PrP 27–30 in the brains of terminally ill Syrian hamsters. (J Am Soc Mass Spectrom 2007, 18, 1070–1079) © 2007 American Society for Mass Spectrometry

Prions are defined as infectious proteins [1], and have been shown to maintain epigenetic states in eukaryotes. In mammals, a prion (PrP^{Sc}) is able to facilitate the conversion of the host's normal cellular protein (PrP^{C}) into PrP^{Sc} and thereby propagate an infection. PrP^{Sc} is believed to be the cause of a group of fatal neurodegenerative diseases called transmissible spongiform encephalopathies (TSEs), known to affect humans and a variety of other animals [2–5]. Detailed studies show that the normal cellular isoform (PrP^{C}) and the infectious isoform (PrP^{Sc}) have identical amino acid sequences and covalent post-translational modifications and, thus, the difference between PrP^{C} and PrP^{Sc} is believed to be exclusively conformational [6–10].

PrP^{C} and PrP^{Sc} have distinct physico-chemical properties. For example, PrP^{C} is rapidly degraded by proteinase K (PK), whereas PrP^{Sc} is much more resistant to PK degradation. The most studied PrP^{Sc} strain is the 263K strain propagated in hamsters. PK digestion of the 263K strain of PrP^{Sc} cleaves the amino terminal domain to give an infectious core consisting primarily of 142 amino acids with an amino terminal Gly90 that is referred to as PrP 27–30 [2]. If the PK digestion of PrP^{Sc} is done in the presence of detergents, rod-shaped particles are obtained, composed of monomers of PrP 27–30. By use of chemical cross-linking and mass spectrometry, the amino terminal Gly90 residues of adjacent monomers in PrP 27–30 rods have been shown to be located within 11 Å of each other [11].

Sensitive prion detection is of importance, not only for research purposes, but also to solve a variety of problems such as the detection of preclinical TSE infections in humans and in animals (e.g., bovines, ovines, cervids), for the assurance of a safe blood supply, and for the early detection of prions in livestock. Prions are present in the blood of infected animals, since the disease has been shown to be transmitted by transfusion [12]. However because of the high specific infectivity of purified prion preparations (as high as 10^{11} infectious units (IU) per milligram of protein purified from Syrian hamsters inoculated with strain 263K) [13], and the fact that in rodent models of TSE, blood contains 5 to 10 IU/mL in preclinical animals and a maximum of about 100 IU/mL at the time of disease onset, it has been estimated that blood contains about 0.05 to 1.0 pg (30–625 amol) of PrP^{Sc} per mL [14].

The incubation-time bioassay in hamster (or transgenic mice) is capable of detecting prions, but since

Address reprint requests to Dr. Bruce Onisko, USDA/ARS/WRRC, 800 Buchanan St., Albany, CA 94710, USA. E-mail: bonisko@pw.usda.gov

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biological assay requires 50 to 270 days, and needs a number of animals, it is not practical for routine testing [15]. Analytical methods have been developed for the detection of PrP^{Sc}. They fall into three categories: Western blots, enzyme-linked immunosorbent assays (ELISAs), and the conformation dependent immunoassay (CDI). The most widely used test is the Western blot, which is reported to detect between 10 to 20 pmol of PrP^{Sc} [16–18]. ELISA methodology is more sensitive, and can detect 2 pmol of PrP^{Sc} [19–21]. The CDI immunoassay is the most sensitive method reported, capable of detection of 0.1 pmol of PrP^{Sc} in brain homogenate (2 ng of PrP of MW 16,241) [22]. Methods to amplify prion titer before detection using either cell culture [23] or cell-free conversion assays [24, 25] show considerable promise. Despite the advancements made in analytical methodology for prion analysis, apparently the detection limit needs to be improved by about three orders of magnitude to allow detection of prions in blood (vide supra, 30 amol/mL in blood versus 0.1 pmol detection by the best antibody methods).

Recently, tandem mass spectrometric methods using nanoscale chromatographic methods have demonstrated selective detection of proteins in biological matrices in sub-femtomole amounts [26, 27]. Furthermore, it has been suggested that the sensitivity of MS/MS detection of peptides may be improved through the use of phenylisothiocyanate (PITC) derivatives, since the MS/MS fragmentation of PITC derivatives gives considerably simplified spectra and thereby may enhance product ion yields [28, 29]. Whereas tandem mass spectrometric methods have been used extensively in the structural characterization of the prion protein [6–10], we are unaware of their use for prion quantitation. We report the results of application of tandem mass spectrometric methods to the problem of detection of the prion protein, and to examine the effect of PITC derivatization on MS/MS sensitivity.

Experimental

Chemicals

Trypsin for in-gel digestion (porcine, sequencing grade, modified) was purchased from Princeton Separations, Adelphia, NJ. Bovine trypsin used for solution digestions was from Sigma (St. Louis, MO). Recombinant Syrian hamster PrP (rPrP) comprising amino acids 90–231 was obtained from InPro Biotechnology (South San Francisco, CA). The peptide VVEQMCTTQYQK, containing an iodoacetamide-derivatized cysteine, was synthesized by Anaspec (San Jose, CA). The structure of this synthetic peptide was confirmed by mass spectrometry; its purity was greater than 90% as shown by LC-UV, and the amount of peptide was quantitated by amino acid analysis. HPLC grade water was purchased from Burdick and Jackson (Muskegon, MI). Acetonitrile, HPLC grade, was from Fisher Scientific (Fairlawn, NJ). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Reduction, Alkylation, and Tryptic Cleavage of Recombinant Syrian Hamster Prion Protein (90–231)

Recombinant PrP containing residues 90–231 (6 nmol) was dissolved in 20 μL of 8 M guanidinium hydrochloride. The mixture was sonicated for 5 min and diluted with 20 μL of 1M TRIS (pH 8.0). To this solution was added 5 μL (1 μmol) of a 200 mM solution of DTT in 1M TRIS (pH 8.0). The reaction mixture was kept at 35 °C for 60 to 90 min. After the reaction cooled to room temperature, 20 μL (4 μmol) of a 200 mM solution of iodoacetamide in 1M TRIS (pH 8.0) was added. The reaction was kept at room temperature in the dark for 60 to 90 min. The excess iodoacetamide was quenched by addition of an additional 25 μL of the DTT solution. The quenched solution was acidified with formic acid to pH 1.5 to 2 (25 μL). The reaction mixture containing the derivatized protein was purified by HPLC using an Alltech Altima C18 column (150 mm × 2.1 mm i.d., 5 μm particle diameter) using a flow rate of 0.25 mL/min and a gradient of 20/80 to 60/40 (vol/vol) of A/B, where solvent A was 0.1% aqueous TFA and solvent B was acetonitrile/isopropanol/TFA (50/50/0.1). One major chromatographic peak was observed (monitoring the UV response at 210 nm) and was collected. The identity and purity of the product was confirmed by LC/ESI/MS. Solvents were removed with a stream of nitrogen. The residue was dissolved in 400 μL water and the pH adjusted to 8.5 by addition of 1M NH_4OH (15 μL). Bovine trypsin (3.3 μg in 33 μL water) was added, and the reaction mixture was kept at 37 °C overnight. The digestion was then stopped by addition of formic acid (2 μL) to give a pH of 2.3.

Derivatization of the rPrP Tryptic Digest with PITC and Edman Cleavage

To 0.5 nmol of the tryptic digest was added 400 μL of a freshly prepared 5% solution of phenylisothiocyanate (PITC) in ethanol/pyridine/water (1:1:1). The reaction mixture was kept at 45 °C for 35 min, cooled to RT and then extracted three times with 1 mL portions of 2:1 heptane/ethyl acetate; organic extracts were discarded. A sample containing about 300 pmol of the PITC-derivatized digest was taken to dryness with a stream of nitrogen. The residue was redissolved in 5 μL of TFA, heated to 40 °C for 10 min, then cooled to RT and diluted with 100 μL of water.

Qualitative Mass Spectrometry: Nanospray LC/MS/MS

NanoLC/ESI/MS/MS was done with an Applied Biosystems (ABI/MDS SCIEX, Toronto, Canada) model

QStar Pulsar equipped with a Proxeon Biosystems (Odense, Denmark) nanoelectrospray source. PrP digest samples (20 μL containing about 1 pmol) were loaded automatically onto a C-18 trap cartridge and chromatographed on a reversed-phase column (Vydac 238 EV5.07515, 75μ × 150 mm; Hesperia, CA) fitted at the effluent end with a coated spray tip (FS360-50-5-CE, New Objective Inc., Woburn, MA). A nanoflow LC system (Dionex, Sunnyvale, CA) with autosampler, column switching device, loading pump, and nanoflow solvent delivery system was used. Elution solvents were: A (0.5% acetic acid in water) and B (80% acetonitrile, 0.5% acetic acid). Samples were eluted at 250 nL/min with the following binary gradient profile: 2% B at 0 min to 80% B in a 15 min linear gradient; held at 80% B for 5 min then back to 2% B for 10 min. The QStar Pulsar was externally calibrated daily and operated above a resolution of 8000. The acquisition cycle time of 6s consisted of a single 1 s MS "survey" scan followed by a 5 s MS/MS scan. Ions between *m/z* 400 to 1000 of charge states between +2 to +5 having intensities greater than 40 counts in the survey scan were selected for fragmentation. The dynamic exclusion window was set to always exclude previously fragmented masses. Collision energy optimized for charge state and *m/z* was automatically selected by the Analyst QS software after adjusting parameters to obtain satisfactory fragmentation of human [Glu^1]-fibrinopeptide B (+2), and human adrenocorticotropic hormone (+3 and +4). Nitrogen was used for the collision gas and the pressure in the collision cell ranged from 3×10^{-6} to 6×10^{-6} torr.

Quantitative Mass Spectrometry: Nanospray LC/MS/MS

NanoLC/ESI/MS/MS was done with an Applied Biosystems (ABI/MDS SCIEX, Toronto, Canada) model 4000 Q-Trap instrument equipped with a nanoelectrospray source. Peptide samples (1 μL containing 1 fmol of human [Val^5]-angiotensin II) were loaded automatically onto a C-18 trap cartridge and chromatographed on a reversed-phase column (Vydac 238EV5.07515, 75μ x 150 mm; Hesperia, CA). A noncoated spray tip (FS360-20-10-N-20-C12, New Objective Inc., Woburn, MA) was used with the Applied Biosystem source, model Microionspray. An LC Packings nanoflow LC system (Dionex, Sunnyvale, CA) with autosampler, column switching device, loading pump, and nanoflow solvent delivery system was used. Elution solvents and gradient profile were the same as described above.

The instrument response was optimized by loop injection of 20 μL of a PrP digest at 1 pmol/μL of 50/49.5/0.5 (acetonitrile/water/acetic acid) onto a reversed-phase column (Vydac 238EV5.07515, 75μ x 150 mm; Hesperia, CA) using isocratic elution of 50/49.5/0.5 (acetonitrile/water/acetic acid) at 250 to 300 nL/min. This method gave stable analyte signals for more than an hour per injection for tuning. Source parameters (electrospray voltage, curtain gas and nebulizing gas settings, ion source heater temperature, declustering potential, and nanospray tip positioning relative to the front plate orifice) were adjusted to obtain maximum analyte signal intensity in Q1MS scan mode while maintaining stable (±5%) electrospray intensity. Fragmentation to obtain specified product ions was optimized by adjusting the Q2 offset voltage ("collision energy").

Syrian Hamster PrP 27–30

A crude preparation of PrP 27–30 was obtained from brains of terminally ill Syrian hamsters inoculated intracranially with the 263K strain of scrapie using modification of a method previously described [30]. Briefly, pellet P145a (as described by Diringer et al. [30]) was obtained, the next two detergent extraction steps were not performed, and pellet P145a was treated with benzonase and proteinase K as done with pellet P145c. The final pellet containing PrP 27–30 was denatured by addition of 6 M guanidinium hydrochloride, and protein was precipitated by centrifugation at 14,000 rpm for 20 min with an Eppendorf model 5417R centrifuge (Hamburg, Germany) after addition of five volumes of methanol at 0 °C.

Electrophoretic Purification and In-Gel Proteolytic Digestion of PrP 27–30

The PrP 27–30 pellet was dissolved with reducing Laemmli sample buffer, boiled for 10 min and subjected to SDS-PAGE [31] using 4% to 12% NuPAGE gels (Invitrogen, Carlsbad, CA). The gel was stained with Coomassie blue, and the major protein bands were excised. In-gel digestion was done with a DigestPro (INTAVIS Bioanalytical Instruments AG, Bergisch Gladbach, Germany). Following washing, reduction with DTT, alkylation with iodoacetamide, and tryptic digestion, the peptides were eluted with 40 ul of 10% formic acid containing 0.1% trifluoroacetic acid.

Results and Discussion

Recombinant PrP (Syrian hamster, amino acid residues 90–231) was reduced with dithiothreitol, alkylated with iodoacetamide, purified by HPLC, then analyzed by nanoLC-ESI-TOF-MS (see Figure 1) to confirm identity of the product. Data in Figure 1 show the presence of one protein product present in charge states ranging from +13 to +22. The *m/z* data shown was transformed using the Bayesian Protein Reconstruct tool in the BioAnalyst ver. 1.1 software (Applied Biosystems, Foster City, CA), which resulted in a measured average molecular weight of 16,357 Da (theoretical average MW of 16,357.1 for $C_{702}H_{1073}N_{211}O_{221}S_{11}$). The reduced and alkylated protein was then digested with trypsin. The tryptic digest, both before and after reaction with phenylisothiocyanate

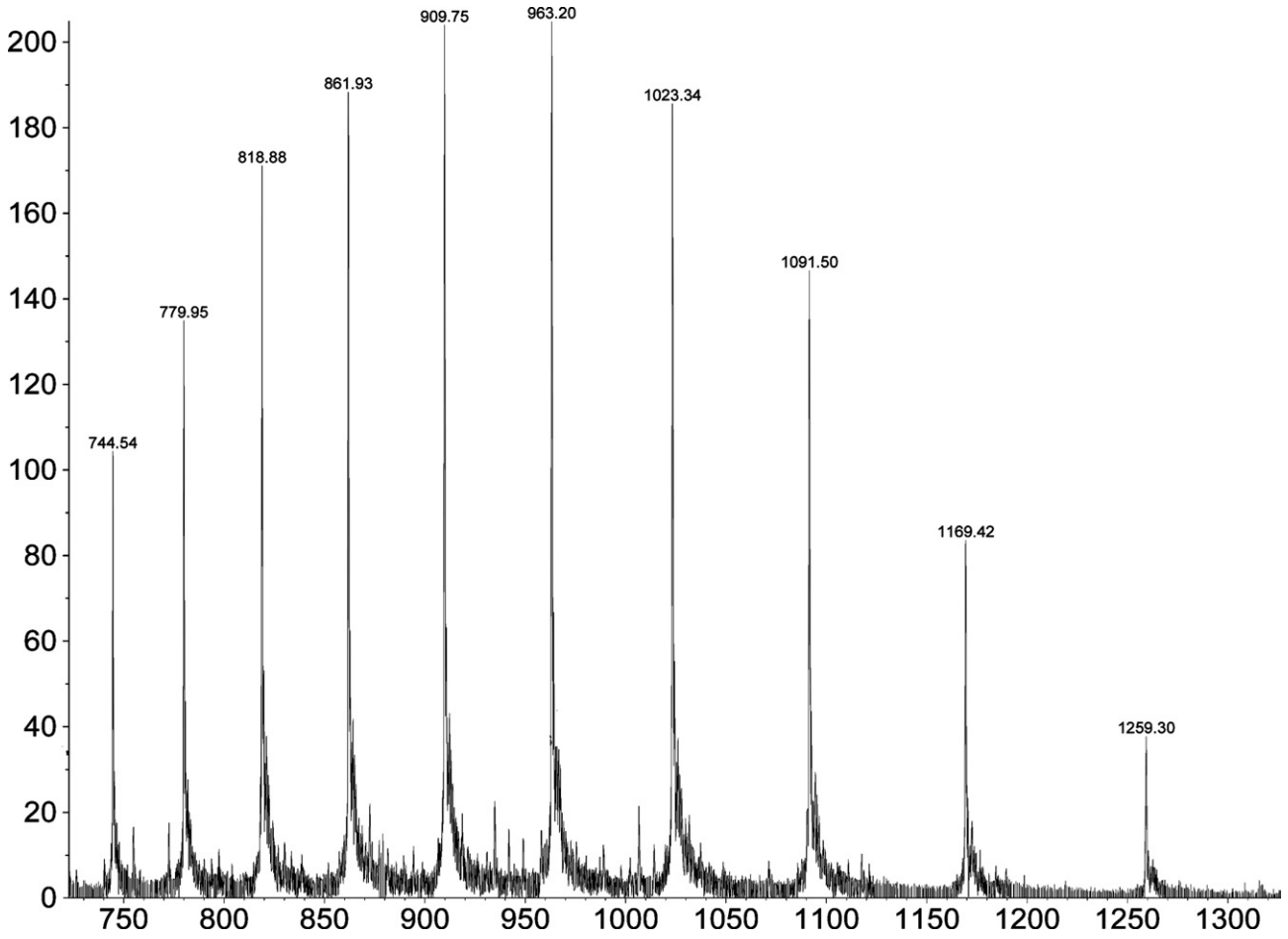

Figure 1. NanoLC/ESI/TOF/MS analysis of reduced and alkylated rPrP (90–231). The x axis is *m/z*.

(PITC), was analyzed by nanoLC/ESI/MS/MS on a quadrupole time-of-flight (Q-TOF) mass spectrometer. The four tryptic peptides that gave the most abundant signal, listed in decreasing abundance, were: GENFTETDIK, ESQAYYDGR, PMMHFGNDWEDR, and VVEQMCTTQYQK. After reaction with PITC, the four derivatized peptides that gave the most abundant signal, also listed in decreasing abundance, were GENFTETDIK, QHTVTTTTK, VVEQMCTTQYQK, and ESQAYYDGR. Proposed structures were confirmed by MS/MS analysis; see Figure 2 and Figure 3 for MS/MS of GENFTETDIK with and without PITC derivatization. Underivatized GENFTETDIK gave a complex (information-rich) spectrum showing excellent coverage of y ions and good coverage of b ions (see Figure 3). PITC-derivatized GENFTETDIK gave a very simple (information-poor) spectrum (see Figure 2) containing primarily the products of gas-phase Edman degradation (b_1 and y_{n-1}), as observed by others with different PITC-derivatized peptides [28, 29]. The dramatically simplified MS/MS spectrum of the PITC derivative suggests that multiple reaction monitoring (MRM) analysis of the PITC derivative using either b_1 or y_{n-1} as the selected product ion may give superior MRM response than the underivatized peptide.

Experiments were done to determine which fragment ion would give the best response for MRM analysis for GENFTETDIK, VVEQMCTTQYQK, and ESQAYYDGR before and after derivatization with PITC. For each peptide, digest samples were directly infused, ionized by nanoESI, and product ion scans were obtained at collision energies ranging from 10 to 110 V. Three product ions were selected for each peptide: the most abundant immonium ion (or fragment thereof), the most abundant b (or a) ion, and the most abundant y (or x ion). Next, instrumental parameters for each transition were optimized. Relative responses for these 18 optimized transitions were obtained by nanoLC/MS/MS of injections of either the underivatized or the derivatized tryptic digest with the mass spectrometer operating in MRM mode. For all three underivatized peptides (Table 1), monitoring the product ion of *m/z* 84, which corresponds to the immonium ion of lysine or glutamine (after loss of NH_3), gave the best detector response. For all three PITC-derivatized peptides (Table 2), MRM using b_1 as the product ion gave the best response.

Direct comparison of the optimized nanoLC-MRM responses of the three tryptic peptides of interest with

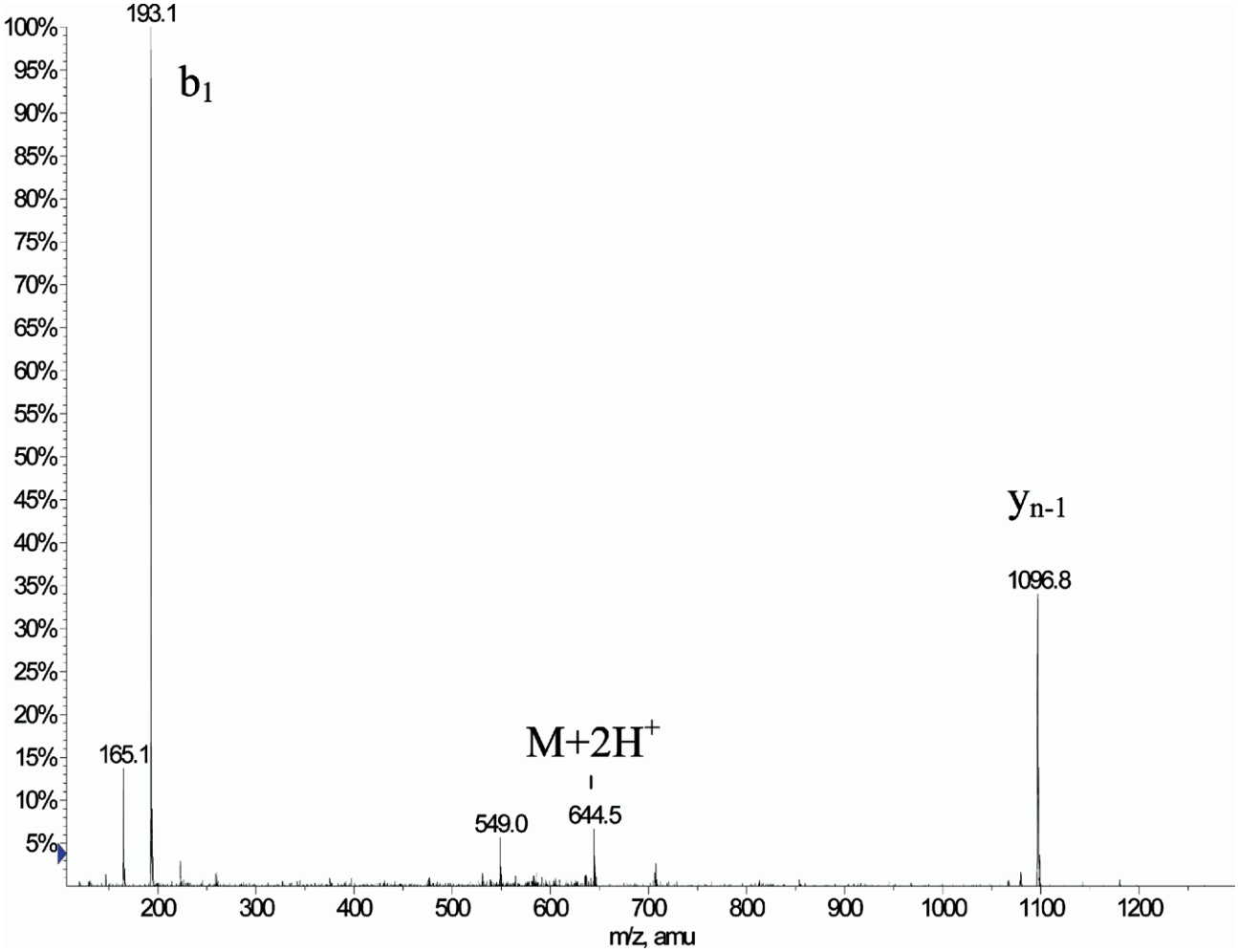

Figure 2. Collision Activated Decomposition (CAD) spectrum of PITC-GENFTETDIK obtained at a collision energy of 22 V.

and without PITC derivatization was done with use of human [Val^5]-angiotensin II (1 fmol/injection) as an internal standard added to each digest. Samples were analyzed by nanoLC/MS/MS, and the MRM responses for the peptides were measured relative to the MRM response of the internal standard, and the MRM response of each underivatized peptide relative to the MRM response of the same peptide, after derivatization was calculated. PITC derivatization gave a diminished MRM response for all three peptides. The largest decrease, about 6-fold, was observed for GENFTETDIK. The MRM response for ESQAYYDGR was 2.5-fold greater than for PITC- ESQAYYDGR, and the MRM response for VVEQMCTTQYQK was 1.3-fold greater than for PITC- VVEQMCTTQYQK.

The decreased MRM responses seen for PITC-derivatized peptides could be the result of chemical instability of the PITC derivatives, decreased ionization efficiency, or decreased MRM efficiency. Potential chemical instability of the PITC derivatives was of concern, since our chromatographic solvents contained dilute acid, and PITC derivatives are known to degrade in acid. To test this possibility, the PITC-derivatized rPrP digest was acidified and heated to release the amino-terminal derivative. This sample was analyzed by nanoLC/ESI/TOF/MS, which confirmed that the desired cleavages had occurred. Furthermore, no PITC-derivatized peptides remained, indicative of complete reaction. ENFTETDIK is the expected degradation product of PITC-GENFTETDIK. An MRM quantitation method for ENFTETDIK was developed on the Q-Trap model 4000 using a precursor ion of *m/z* 547.7, a product ion of *m/z* 706.4 and a collision energy of 28 V. Using this method, and assuming a quantitative yield of ENFTETDIK from PITC-GENFTETDIK, the amount of ENFTETDIK in the PITC-derivatized rPrP digest was determined. For injections of 1.2, 30, and 150 fmol of the PITC-derivatized rPrP digest, ENFTETDIK, as a percentage of the amount injected, was found to be 2.2%, 2.6%, and 2.5%, respectively. Clearly, decomposition of the PITC-derivatized peptide, either before or during analysis, is not a significant problem.

MRM efficiency was estimated by calculating the percentage of the total ion current (TIC) present as the

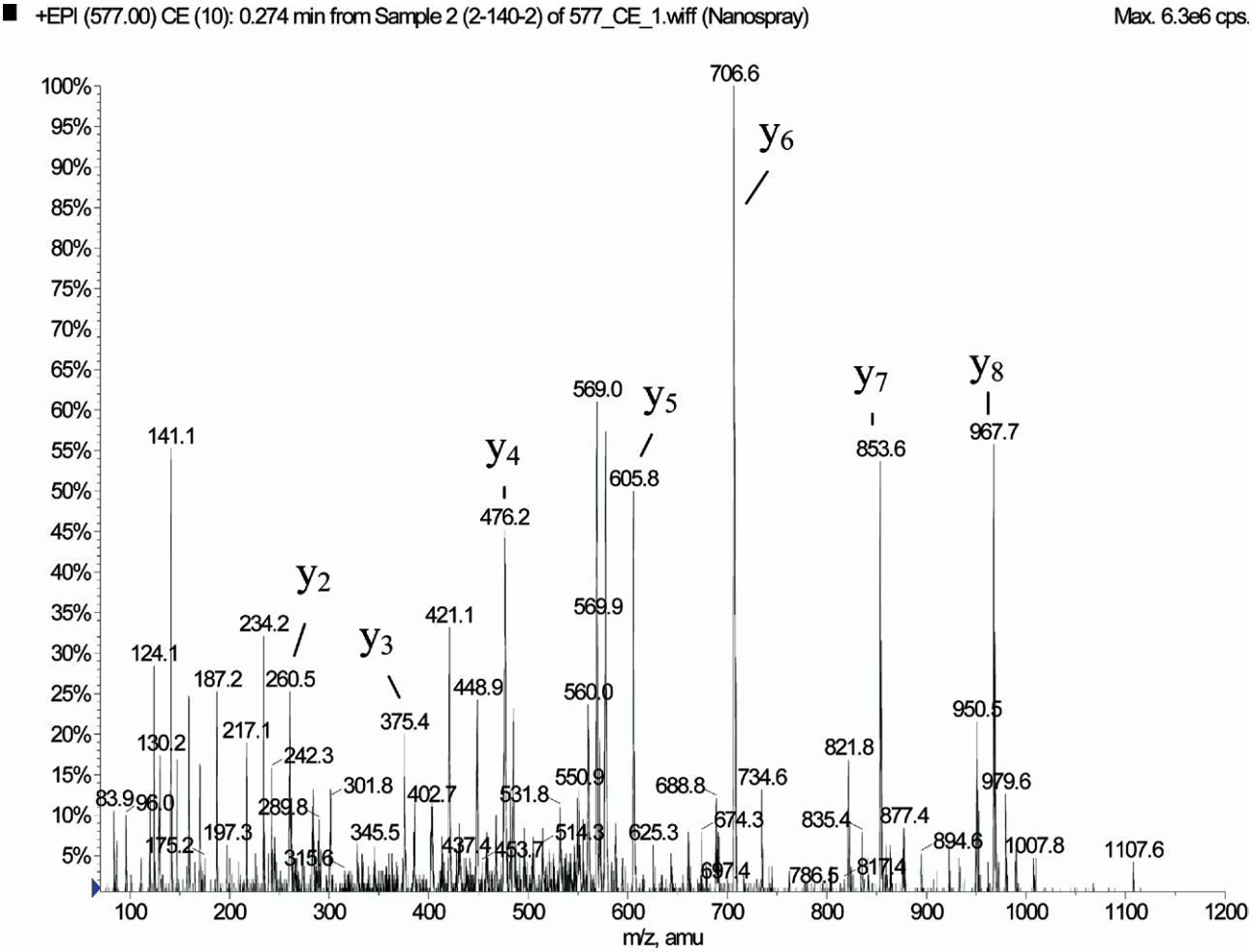

Figure 3. CAD spectrum of GENFTETDIK obtained at a collision energy of 30 V.

product ion of interest using optimized instrumental parameters. For example, for PITC-GENFTETDIK (see Figure 2), the b_1 product ion of *m/z* 193 monitored by MRM represents 33% of the TIC. For GENFTETDIK, the product ion of *m/z* 84 monitored by MRM under conditions optimized for detection of this fragment (see Figure 4) was 24%, quite similar to that observed for the b_1 ion from the PITC derivative.

Thus, neither chemical instability of PITC derivatives nor diminished MRM efficiencies of the PITC-peptides can explain the observation that the nanoLC/ESI/MRM response of PITC-derivatized peptides was significantly less than that of the underivatized peptides. Presumably, the PITC derivatives have diminished ESI ionization efficiency. This has indeed been observed to be true for MALDI ionization of PITC derivatives [32]. This problem might be solved by using modified Edman reagents designed for enhanced ESI ionization efficiency of the product phenylthiohydantoins used in Edman sequencing [33].

Table 1. Optimized MRM response for selected tryptic peptides from rPrP

		Optimized MRM for best y (or x) ion			Optimized MRM for best b (or a) ion			Optimized MRM for best immonium ion		
Peptide	z = 2 precursor (*m/z*)	product ion (*m/z*)	CE (V)	Relative response[a]	Product ion (m/z)	CE (V)	Relative response[a]	product ion (*m/z*)	CE (V)	Relative response[a]
GENFTETDIK	577.3	706.4(y6)	30	40+/−3	1007.4(b9)	20	2.6+/−0.3	84.1[b]	100	100+/−5
ESQAYYDGR	544.7	673.3(y5)	28	15+/−2	579.2(b5)	22	1.0+/−0.2	84.1[b]	100	18+/−3
VVEQMCTTQYQK	757.8	438.2(y3)	35	1.3+/−0.2	171.1(a2)	50	4.8+/−0.3	84.1[b]	100	6.3+/−0.8

[a]Response relative to the most abundant (100) is given as the mean +/− SD (n = 8).
[b]The ion of *m/z* 84.1 is the immonium ion of K (or Q) after loss of NH_3.

Table 2. Optimized MRM response for selected tryptic peptides of rPrP derivatized with PITC

		Optimized MRM for $y_{(n-1)}$ ion			Optimized MRM for b_1 ion			Optimized MRM for best immonium ion		
PITC-peptide	z = 2 precursor (*m/z*)	Product ion (*m/z*)	CE (V)	Relative response[a]	Production (*m/z*)	CE (V)	Relative response[a]	Product ion (*m/z*)	CE (V)	Relative response[a]
GENFTETDIK	644.8	1096.5 (y9)	22	79+/−8	193.0 (b1)	25	100+/−10	120.1[b]	85	16+/−2
ESQAYYDGR	612.2	959.4 (y8)	20	32+/−4	265.1 (b1)	20	42+/−6	102.1[b]	56	15+/−2
VVEQMCTTQYQK	825.4	1415.6 (y11)	32	16+/−2	235.1 (b1)	32	29+/−4	101.1[b]	90	2.2+/−0.3

[a]Responses are given as mean +/− SD (n = 4).
[b]Immonium ions for the three peptides in descending order are from F, E, and Q, respectively. (Theoretical *m/z* for Q is 101.071; for K is 101.107; *m/z* of 101.072 was observed on the Q-Star from MS/MS of VVEQMCTTQYQK).

Because of the superior MRM responses for the underivatized peptides, a PrP quantitation method was established without use of PITC. Of the three tryptic peptides that gave the best MRM sensitivity, VVEQMCTTQYQK was chosen for quantitation of the prion protein. GENFTETDIK was excluded because asparagine 197 is modified via N-linked oligosaccharides in the majority of naturally-occurring PrP molecules. ESQAYYDGR was also excluded since the peptide is absent in a carboxy-truncated species (about 10% to 15% of total PrP) as observed by others [10]. This leaves VVEQMCTTQYQK by process of elimination. (PMMHFGNDWEDR is another good candidate, but the presence of two methionines makes possible four isomeric monosulfoxides (two positional and two diastereomeric) and two isomeric disulfoxides (diastereomeric). For VVEQMCTTQYQK, the a2 product ion of *m/z* 171 was chosen for MRM detection instead of the somewhat

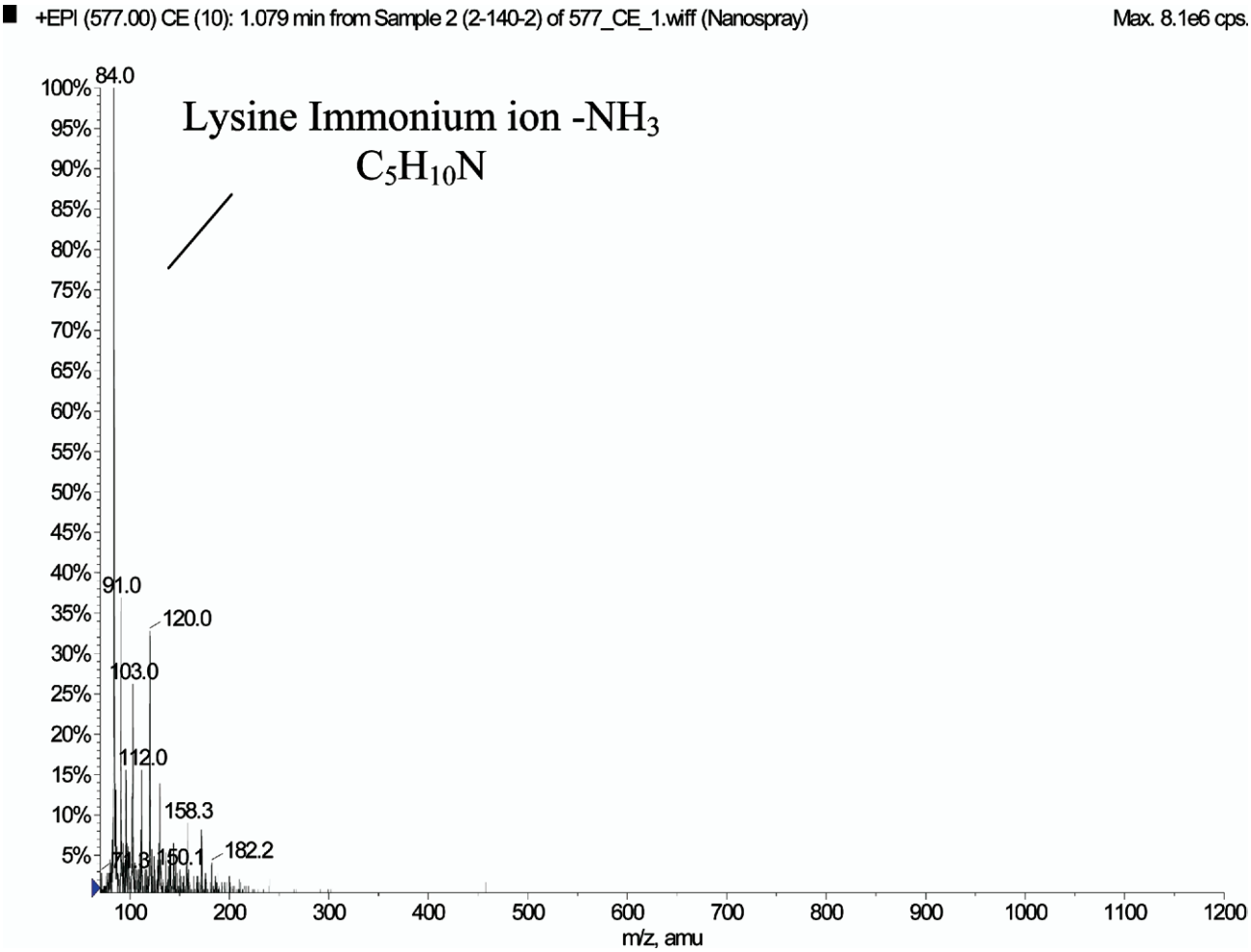

Figure 4. CAD spectrum of GENFTETDIK obtained at a collision energy of 100 V.

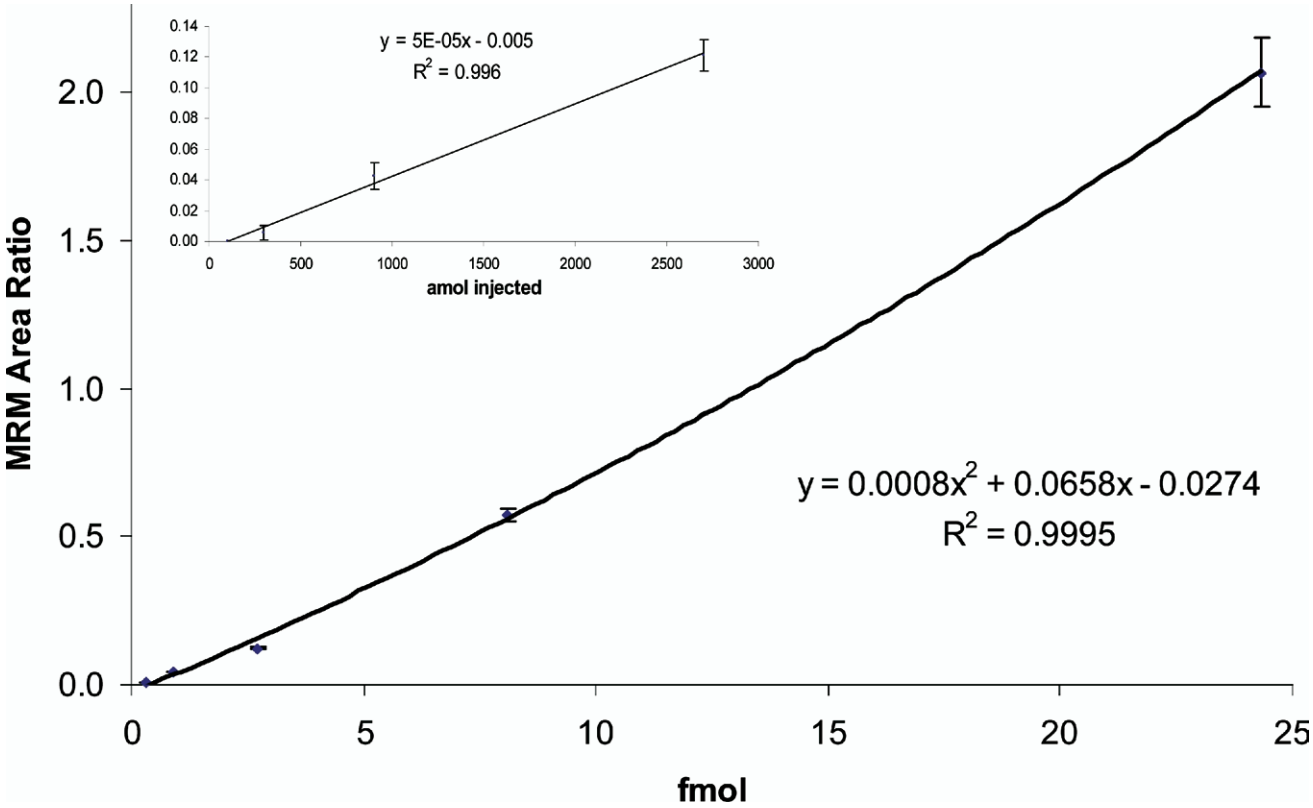

Figure 5. Area of MRM response of VVEQMCTTQYQK to the MRM response of the internal standard (1 fmol human [Val^5]-angiotensin II) versus amount of VVEQMCTTQYQK injected. Error bars show the mean ± standard deviation (n = 5). The insert is an expansion of the results from the three lowest amounts injected. Data in the insert show the mean ± 3 * (standard deviations).

more abundant 84 ion (see Table 1), since the ion of m/z 84 is expected from any peptide containing either lysine or glutamine, whereas the a2 ion should only be seen in MS/MS of peptides containing amino terminal VV, PK, or KP, only three of 400 possible dipeptides. A calibration curve using the optimized 757.8 to 171 transition (see Figure 5) was obtained with synthetic VVEQMCTTQYQK, the amount of which had been quantitated by amino acid analysis, and using human [Val^5]-angiotensin II (1 fmol/injection) as internal standard. The results show a dynamic range of at least three orders of magnitude. Response is reasonably linear; however the data more closely fit a second-order polynomial, as shown. MRM responses for pentuplicate injections of 300 to 2700 amol are shown as an insert in Figure 5. The results for this range are linear (r^2 of 0.996). Error bars in the insert are displayed as +/− three times the calculated standard deviation for visual clarity. The chromatographic profile for 0 (solvent only), 300, and 900 amol of VVEQMCTTQYQK is displayed in Figure 6. Signal to noise (S/N) was calculated using the standard deviation of the MRM response before elution of the analyte as an estimate of instrumental noise. For the pentuplicate injections of 900 amol of VVEQMCTTQYQK, a mean S/N of 126 was observed, from which a limit of detection (LOD) (defined as the amount that should give a $S/N = 3$) of 20 amol can be calculated. For the pentuplicate injections of 300 amol of VVEQMCTTQYQK, a mean S/N of 33 was observed, from which a LOD of 30 amol can be calculated for detection of the prion protein. The LOD of 20 to 30 amol achieved by nanoLC/MRM is nearly four orders of magnitude more sensitive than the 0.2 pmol sensitivity reported for the most sensitive antibody-based detection method (vide supra) [22].

A crude preparation of PrP 27–30 was obtained from the brains of terminally ill Syrian hamsters inoculated intracranially with the 263K strain of scrapie. The sample preparation comprised tissue homogenization, centrifugation to obtain a sarkosyl-insoluble pellet, treatment with benzonase and proteinase K, then further purification by SDS-PAGE. Three bands are seen for PrP 27–30 by SDS-PAGE and are known to correspond to molecular species containing 0, 1, or 2 N-linked glycans per molecule. Our SDS-PAGE separation clearly resolved the band containing no N-linked glycans from the other two bands that were poorly resolved (data not shown). Subsequently, we excised only two gel regions: one containing PrP with no N-linked glycans and the other containing PrP with either one or two N-linked glycans. Excised gel regions were destained, reduced, alkylated, treated with trypsin, the resulting peptides were extracted, and the amount of

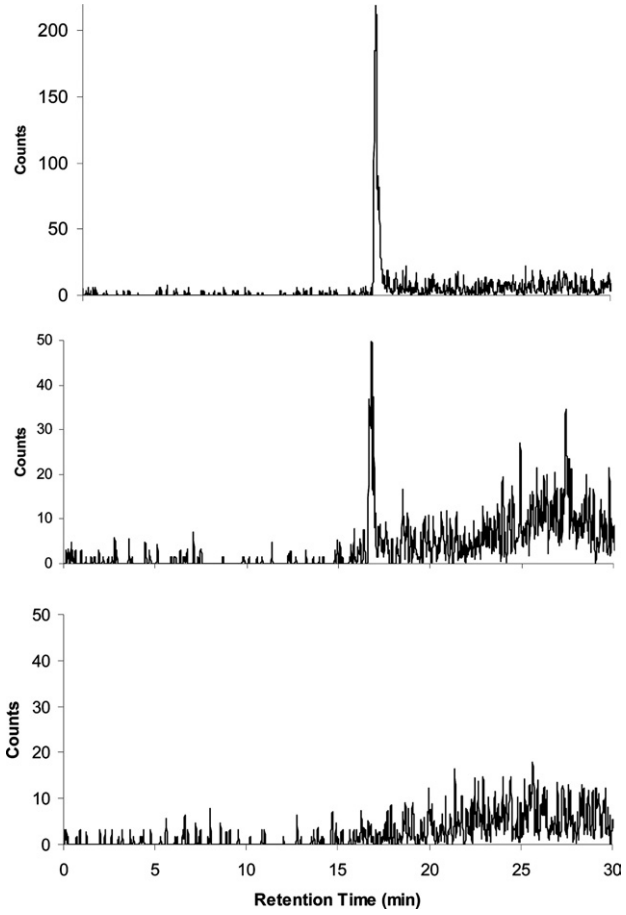

Figure 6. Elution profile (MRM response) for injection of 0, 300, and 900 amol of VVEQMCTTQYQK. Data has been smoothed, but S/N and LOD in the text were calculated using raw data.

VVEQMCTTQYQK was quantitated using the method described (vide supra).

Recovery experiments were performed to estimate the overall yield of the chemical, enzymatic, and physical steps used after isolation of crude PrP 27–30. Various amounts of rPrP (quantitated by OD at 254 nm) were subjected to SDS-PAGE, in-gel digestion, and quantitation of VVEQMCTTQYQK by nanoLC/ESI/MRM as done for PrP 27–30. The results, shown in Figure 7, show overall recovery of VVEQMCTTQYQK from rPrP ranging from 9% to 33%, depending on the amount of starting rPrP.

The amounts of VVEQMCTTQYQK obtained from the excised gel pieces from SDS-PAGE of crude PrP 27–30 were corrected using the estimated recoveries shown in Figure 7, and the results are shown in Table 3. We estimate 19 ug/g of brain (wet weight) of PrP 27–30 in terminally ill hamsters, composed of 1.2 ug/g of PrP 27–30 containing no N-linked glycans and 17 ug/g of PrP 27–30 containing N-linked glycans. This result is in excellent agreement with the estimate by Diringer et al. [30] of 21 ug/g of PrP 27–30.

Using pure standards, it is straight forward to compare the LOD for our method (30 amol) to that of the

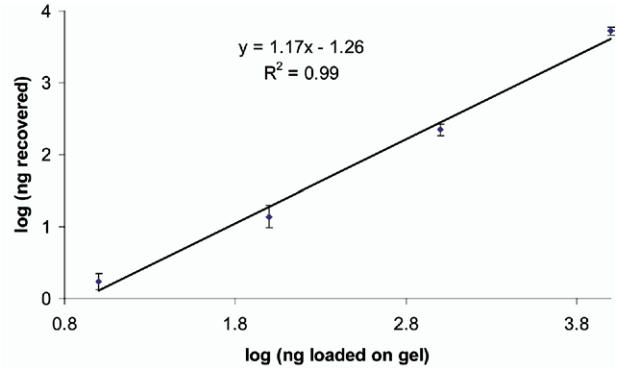

Figure 7. Recovery of VVEQMCTTQYQK after SDS-PAGE purification, reduction, alkylation, and tryptic cleavage of recombinant Syrian hamster PrP comprising amino acids 90–231.

CDI (300 amol, calculated from 5 pg protein of MW 16,240) [22]; the mass spectrometric method appears about ten times more sensitive. Detection of prions in biological materials is much more difficult, and a comparison of the LOD of our method with the CDI starting with brain can be estimated. To obtain the results for PrP 27–30 shown in Table 3, crude PrP 27–30 from 0.57 g brain (wet weight) was loaded on each SDS-PAGE lane, the major band was excised and processed as described by in-gel digestion to give 150 uL of digest. After a 10-fold dilution of this digest, 1 uL was injected onto the nanoLC column, and the MRM response (raw data not shown) gave a single peak of the correct retention time with an average S/N for triplicate injections of 2250. This corresponds to a calculated LOD of 110 amol for PrP 27–30 from brain, about three times higher than the LOD found with pure standard peptide, presumably because of the 33% recovery obtained with this amount of material (see Figure 7). For ng quantities in brain samples, our % recovery should be about three times less (see Figure 7), so we estimate the LOD for detection of ng amounts of PrP 27–30 from brain to be about 300 amol, which is about 300 times more sensitive than the reported LOD of 0.1 pmol for the CDI using rPrP in brain homogenate (2 ng of PrP of MW 16,240) [22].

In conclusion, we have developed a mass spectrometric method for quantitation of the prion protein and demonstrated its use in detection of PrP 27–30 in the brains of terminally ill Syrian hamsters. This method is appropriate for detection of PrP^{Sc}, and has better sensitivity than any published antibody-based method.

Table 3. Quantitation of PrP 27-30 molecular species in brains of terminally ill Syrian hamsters

Molecular species	Amount ug/g brain)[a]
PrP 27-30 with no N-linked glycans	1.2+/−0.2(3)
PrP 27-30 with either 1 or 2 N-linked glycans	17+/− 2(3)
Total	19+/− 2(3)

[a]Mean +/− the standard deviation of triplicate analyses.

Future studies will focus on sample preparation for attempts to detect PrP^{Sc} in blood.

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