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Transmissible spongiform encephalopathy strain-associated diversity of N-terminal proteinase K cleavage sites of PrP^{Sc} from scrapie-infected and bovine spongiform encephalopathy-infected mice

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Abstract

Assessment of the different conformational states of the abnormal prion protein (PrP^{Sc}) in the CNS provides an established basis for distinguishing transmissible spongiform encephalopathy (TSE) strains. PrP^{Sc} conformers are variably resistant to N-terminal proteinase K (PK) digestion, and analysis of the consensus products (PrP^{res}) by immunoassay enables effective, but relatively low-resolution differentiation. Determination of the precise N-terminal amino acid profile (N-TAAP) of PrP^{res} presents a potential high-resolution means of TSE-strain typing, and thus of differential disease diagnosis. This approach was evaluated using individual mice affected by model scrapie (22A, ME7, 87V and 79A) and bovine spongiform encephalopathy (BSE) (301V) strains. Nano liquid chromatography–mass spectrometry (LC-MS) was used to determine PrP^{res} N-terminal tryptic digestion products. Four major N-terminal tryptic peptides were generated from all mouse TSE strains investigated, corresponding with predominant N-termination of PrP^{res} at G^{81}, G^{85}, G^{89} and G^{91}. Both the mass spectrometric abundance of the individual peptides and the ratios of pairs of these peptides were evaluated as markers of conformation in relation to their potential for strain discrimination. The yield of peptides was significantly greater for BSE than scrapie strains and the relative quantities of particular peptide pairs differed between strains. Thus, whereas peptide G^{91}–K^{105} was a dominant peptide from 301V, this was not the case for other strains and, significantly, the ratio of peptides G^{91}–K^{105}:G^{89}–K^{105} was substantially higher for BSE-infected compared with scrapie-infected mice. These data support the potential of the N-TAAP approach for high-resolution TSE strain typing and differential diagnosis.

Keywords: *Transmissible spongiform encephalopathy, prion, conformation, strain, differential diagnosis, mass spectrometry*

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Introduction

Transmissible spongiform encephalopathies (TSEs) are a family of degenerative diseases of the central nervous system that include scrapie, bovine spongiform

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encephalopathy (BSE), chronic wasting disease in deer and elk and Creutzfeldt–Jacob disease (CJD) in humans. They can occur sporadically, be linked to mutations in the prion protein (PrP) gene, or be acquired by infection (e.g. iatrogenic CJD) and there is evidence that a new variant of CJD has resulted from the consumption of BSE-contaminated food (Will et al. 1996).

According to the 'prion hypothesis' (Prusiner 1982) the infectious form of the prion protein has an identical amino acid sequence to the normal cellular prion protein PrP^{c}, and differences between the spongiform encephalopathy phenotypes are derived solely from differences in PrP conformation following a post-translational transition (Stahl et al. 1993). This involves transition of the predominantly α-helical structure of PrP^{c} into PrP^{sc}, which is rich in β-sheet, especially in the region ~90–145 (Pan et al. 1993, Huang et al. 1996, Jackson et al. 1999). N-terminal sequencing and mass spectrometry (MS) have enabled identification of two forms of PrP^{Sc} in different human prion diseases, which can be used to aid differentiation. These can be distinguished by the size of the proteinase K (PK)-resistant core fragment as a consequence of difference in primary N-terminal cleavage sites: predominantly at residue 97 in the case of fatal familial insomnia and at residue 82 in CJD and Gerstmann–Straussler–Scheinker syndrome (Chen et al. 2000, Parchi et al. 2000). The ragged nature of the PK-cleaved N-terminus is evident from the numerous secondary cleavage sites along the region spanning residues 74–102. Accordingly, in terms of PK susceptibility, the PrP^{Sc} structure can be delineated by three regions, the N-terminus (residues 23–73) which is invariably PK sensitive, a PK-resistant C-terminal region (residues ~103–231), and, third, residues 74–102 which may be considered as variably resistant to PK, probably reflecting the extent of the β-sheet structure. Similar strain-related differences in PK cleavage site are also evident for BSE and scrapie PrP^{Sc} using Western blot (WB) techniques (Stack et al. 2002) and for mouse-passaged BSE and scrapie using Edman sequencing (Hayashi et al. 2005); the latter approach presents the possibility of high-resolution strain typing of prion diseases. Current biochemical methods of prion disease classification are reliant on relatively low-resolution procedures such as WB to discriminate through identification of distinct differences in mass of mono- and diglycosylated and unglycosylated isoforms of PrP^{res} (Collinge et al. 1996, Somerville et al. 1997, Stack et al. 2002), and on immunohistochemical examination using antibody panels to profile distinct patterns of distribution and accumulation of disease-specific PrP variants (Jeffrey et al. 2001, Gonzalez et al. 2003). Each of the three glycoforms is likely to represent a consensus PrP^{res} exhibiting, in strain-dependent proportions, various PK cleavage sites between residues ~74–102. Although WB analysis can differentiate strains, it cannot readily resolve the full range of N-terminal cleavage products associated with the individual glycoforms. The extent to which these cleavage sites vary subtly between strains remains to be established but, given a sufficiently sensitive, accurate and reproducible method, N-terminal amino acid profiling (TAAP) of PrP^{res} should enable robust high-resolution differential diagnosis of TSE strains such as scrapie and BSE in sheep.

The aim here was to develop a liquid chromatography (LC)-MS-based analytical strategy capable of establishing, in individual subjects, the nature and extent of variation of N-terminal PK cleavage sites of PrP^{Sc} to the level of individual amino acid residues. Nano LC-electrospray-MS was thus applied to sequence the PrP^{res} N-terminus following tryptic digestion, using murine models to evaluate the potential of

this approach for strain differentiation. To date, in the prion protein field, LC-MS-based sequencing approaches have been applied largely to targeted identification of prion protein allotypes and potential post-translational modifications (Gill et al. 2000, Schinina et al. 2003, Cartoni et al. 2005). These, and other MS-based studies of PrP^{Sc} structure (Stahl et al. 1993) required pooling of brain material to isolate sufficient PrP^{Sc}, and this has hitherto hindered ready investigation of biological variation in cleavage point susceptibility. Here, the balance between PrP^{res} recovery and purity were achieved by sequential application of two widely established complementary isolation techniques: precipitation with sodium phosphotungstic acid (NaPTA) (Safar et al. 1998) and centrifugation through a sucrose cushion (Hope et al. 1988).

Materials and methods

Chemicals and reagents

All chemicals and reagents were of reagent grade or high-performance LC (HPLC) gradient grade (Riedel de Hahn, Chromasolve) and were obtained from Sigma-Aldrich (Poole, UK), unless otherwise stated. Trypsin (V511, >5000 u mg^{-1}) was from Promega (Madison, WI, USA). Prionics buffer and proteinase K (PK solution) were part of the 'Prionics-Check' PrP^{Sc} detection kit and were prepared as recommended (Prionics AG, Schlieren, Switzerland). 'Iodide solution' contained potassium iodide (0.9 M), sodium thiosulphate (9 mM), sodium phosphate (15 mM, pH 8) and sarkosyl (1% w/v), and 20% sucrose/iodide solution was prepared by dissolving sucrose (10 g) in iodide solution (33 ml) and diluting to 50 ml with water. Phosphate-buffered saline (0.9% NaCl) was prepared in 0.05 M sodium phosphate, pH 7.0.

Infection of mice with TSE strains and isolates

Mice were inoculated via the intracerebral route with brain homogenate (10% w/v in sterile normal saline, 0.02 ml) prepared from either whole murine brain, in the case of passaged strains (301V, ME7, 22A, 79A, 87V) or, for isolates, from pooled brains from BSE-infected cattle or scrapie-infected sheep (Table I). Control mice were similarly maintained but not inoculated. Following clinical observation of disease, mice were euthanased and brains excised and stored at –80°C until required. Individual whole brain samples ($n=4$) from each strain/isolate inoculum group were selected randomly for PrP^{res} N-terminal sequencing.

Isotyping of PrP^{res} by Western blot analysis

For reference purposes PrP^{res} isoform profiles for each 'strain' were evaluated by WB of a single brain sample from each group, using 6H4 antibody for detection (Stack et al. 2002).

Isolation of PrP^{res} from mouse brain

PrP^{res} was isolated using a combination of NaPTA precipitation (Safar et al. 1998) and sucrose cushion centrifugation (Hope et al. 1988). Individual brains (0.4 ± 0.04 g) were homogenized (Omni GLH, Camlab, Cambridge, UK) in Prionics buffer (final

Table I. Incubation periods for various TSE strains and isolates following intracerebral inoculation of mice with brain homogenate.

Mouse strain	Genotype	Inoculum	Source of TSE infected brain inoculum	Days to clinical disease Mean ± SD (*n*)
VM	prnp^{b}	22A	Mouse-passaged scrapie	204 ± 7 (20)
C57BL	prnp^{a}	79A	Mouse-passaged scrapie	164 ± 3 (20)
VM	prnp^{b}	87V	Mouse-passaged scrapie	322 ± 27 (20)
C57BL	prnp^{a}	ME7	Mouse-passaged scrapie	175 ± 7 (20)
RIII	prnp^{a}	Field case scrapie	Primary-passaged ovine scrapie isolate	480 ± 40 (24)
VM	prnp^{b}	301V	Mouse-passaged BSE	110 ± 3 (25)
RIII	prnp^{a}	Field case BSE	Primary-passaged bovine BSE isolate	483 ± 35 (25)

The prion gene of mice (*prnp*) has two well-characterized alleles, prnp^{a} and prnp^{b}, differing in sequence at codons 108 and 189, and represented by amino acids (108^{L} and 189^{T}) and (108^{F} and 189^{V}), respectively.

volume 4 ml), then centrifuged at 2000*g* for 2 min. The supernatants were incubated (0.5 h, 50°C) with benzonase solution (E1014, 250 units μl^{-1}; 0.2 ml containing 200 units in 20 mM $MgCl_2$) and then with PK solution (0.4 ml, 1 mg ml^{-1}) for 1 h (50°C), after which the protease was deactivated by heating (15 min) in a boiling water bath. Protein was precipitated by treatment with NaPTA (4% w/v in 170 mM $MgCl_2$, 0.32 ml) for 30 min at 37°C with agitation then centrifuged (22 000*g*, 1 h). Supernatants were discarded, and the pellets resuspended in 0.4 ml sarkosyl (0.1% w/v in phosphate-buffered saline, pH 7.4) and EDTA (0.1 ml, 250 mM). The suspensions were centrifuged (22 000*g*, 30 min), the supernatants discarded and pellets stored at −80°C. Further purification was carried out essentially as described (Hope et al. 1988). Pellets were suspended in water (1.25 ml) and iodide solution (2.5 ml), transferred onto a cushion of 20% sucrose/iodide solution (1.25 ml) in a 5 ml tube and centrifuged at 287 000*g* (av.) for 1.5 h at 10°C (Beckman L8-60 centrifuge, SW55Ti swing out head). The supernatants were discarded and the PrP^{res} pellets washed with water (0.5 ml) and recovered by centrifugation (9000*g* for 0.5 h). PrP^{res} was then analyzed directly or stored at −80°C.

Reduction and alkylation of PrP ^{res}

The PrP^{res} pellet was solubilized in guanidine hydrochloride (50 µl, 6 M in 50 mM Tris, pH 8.0), reduced with 2 mM dithiothreitol (5 µl) at 95°C (0.4 h) and after cooling, alkylated with 4-vinyl pyridine (6 mM, 5 µl) at ambient temperature for 1.5 h (Stimson et al. 1999). Following centrifugation (8000*g*, 2 min) the supernatant was recovered (discard pellet) and the protein isolated by precipitation with −20°C methanol (0.25 ml) and maintaining at −20°C for at least 10 min before centrifugation (15 000*g*, 10 min) at 4°C. The supernatant was discarded and the pellet resuspended in −20°C methanol, centrifuged at 15 000*g* for 2 min at 4°C and, after discarding the supernatant, the pellet was allowed to dry at ambient temperature.

Tryptic digestion of PrP^{res}

The dried PrP^{res} pellet was suspended in freshly prepared urea (6 M, 10 µl) then Tris/methylamine solution (10 µl: 150 mM Tris buffer, pH 8.0 containing 60 mM methylamine and 15 mM calcium acetate) and the synthetic trypsin substrate boc-val-leu-lys-7-amido-4-methylcoumarin (bocVLK-AMC, 4 ng in 2 µl) were added. Digestion was initiated at 30°C by addition of trypsin solution (0.1 µg ml^{-1}, 2 µl). The reaction was terminated after 18 h by reducing the pH to below 2 (20% v/v heptafluorobutyric acid (HFB), 2 µl). Digests were stored at −20°C until analyzed.

Nano liquid chromatography

Tryptic digest samples (10 µl) were transferred onto a prewashed (0.1% HFB, 200 µl) reverse phase precolumn (cap-trap, 004/25108/31, Michrom Bioresources, CA, USA) using an autosampler (FAMOS, Dionex UK Ltd, Camberley, UK) linked to a column switching valve system (Switchos, Dionex). Urea and other residual polar reagents and buffers were washed from the precolumn (0.02% HFB in 0.1% formic acid, 60 µl), before sample elution at 0.3 µl min^{-1} (Ultimate MS pump, Dionex) to the nano capillary column (65 × 0.075 mm id column, PicoFrit™ PF360-75-8-N-5, New Objective, Woburn, USA, packed with 'Jupiter Proteo' stationary phase (Phenomenex, Macclesfield, UK) (Howells et al. 2005). A solvent gradient was used, ranging from 95% solvent A (0.1% formic acid in 0.5% acetonitrile) and 5% solvent B (0.1% formic acid in acetonitrile) over the first 2 min to 30% B at 25 min. The column was washed (70% B for 5 min) and re-equilibrated (5% B for 20 min) before the next injection. During re-equilibration, the precolumn was washed with 90% acetonitrile in 0.1% formic acid (250 µl). Solvents for the washing and prewashing of the precolumn (flow rate 10 µl min^{-1}) were delivered through a flow splitter (Acurate™, LC Packings, the Netherlands; split from 0.3 ml min^{-1}).

Ion trap mass spectrometry and data collection

Peptides were eluted into the ion trap MS (LCQ Classic, Thermofinnigan, Hemel Hempstead, UK) via a nanoelectrospray source (Protana; MDS Proteomics Inc, Toronto, Canada). The capillary interface temperature was set to 150°C and the source voltage at the column outlet set to 2.5 kV. Data were collected from 0 to 30 min using a data-dependent algorithm whereby the column eluant was scanned continuously between m/z 190 and 2000; MS^2 spectra were obtained automatically in real time for the two most intense ions above a threshold of 1×10^5 counts from each full scan. In addition, preferential MS^2 fragmentation was performed against a list of selected (observed) peptide ion masses as follows: 459.2 (bocVLK), 727.1, 781.2, 818.7, 852.0, 866.8, 911.6, 1166.4 (see Table II).

Data analysis

Amino acid residues are numbered according to the murine PrP sequence (Westaway et al. 1987). Tryptic peptides were identified using the SEQUEST programme (Thermofinnigan) using a database of murine and ovine PrP sequences. The quality of match between experimentally obtained and software-predicted MS^2 spectra is indicated by the calculated cross-correlation scores (Xcorr). Scores >2.7 for partially

Table II. Tryptic peptides of brain PrP^{res} from a mouse infected with BSE (301V).

Retention time (min)	Amino acid sequence[a]	Ions detected (charge states)	Relative abundance[b] [charge state selected]	m/z calculated $[M+nH]^{n+}$	m/z observed $[M+nH]^{n+}$	Xcorr value	MM_{avg} (Da)
13.3	73–105	$2^+,3^+,4^+$	9.1 $[4^+]$	852.2	852.0	(-)	3404.6
13.6	77–105	$2^+,3^+,4^+$	4.5 $[4^+]$	747.3	747.4	4.3	2985.2
13.7	79–105	$2^+,3^+,4^+$	10 $[3^+]$	958.0	957.9	4.5	2871.1
13.4	80–105	$2^+,3^+,4^+$	3.0 $[3^+]$	929.0	929.0	3.8	2784.0
11.3	81–105	$2^+,3^+,4^+$	100 $[3^+]$	866.9	866.8	3.9	2597.8
11.5	83–105	$2^+,3^+,4^+$	2 $[3^+]$	805.2	805.2	(-)	2412.6
11.8	85–105	$2^+,3^+,4^+$	29 $[3^+]$	727.1	727.1	3.7	2178.3
11.7	86–105	$2^+,3^+,4^+$	3.7 $[2^+]$	1061.6	1061.3	3.3	2121.3
11.7	87–105	$2^+,3^+,4^+$	6.2 $[2^+]$	1033.1	1032.7	2.9	2064.2
11.3	88–105	$2^+,3^+,4^+$	7.6 $[2^+]$	1004.6	1004.3	(-)	2007.2
9.7	89–105	$1^+,2^+,3^+,4^+$	99 $[2^+]$	911.5	911.6	2.8	1821.0
9.6	90–105	$2^+,3^+,4^+$	1.4 $[2^+]$	883.0	883.0	(-)	1763.9
9.5	91–105	$1^+,2^+,3^+,4^+$	79 $[2^+]$	818.9	818.7	2.7	1635.8
9.5	92–105	$1^+,2^+,3^+,4^+$	3.05 $[2^+]$	790.4	790.7	(-)	1578.7
23.6	110–135	$2^+,3^+$	125 $[2^+]$	1166.9	1166.4	4.9	2331.7
17.4	136–147	$1^+,2^+,3^+$	56 $[3^+]$	506.5	506.6	4.4	1516.6
9.3	151–155	1^+	156 $[1^+]$	712.8	712.3	1.04	711.8
12.2	194–203	2^+	41.7 $[2^+]$	570.6	570.6	2.9	1139.2
12.7	208–219	$1^+,2^+,3^+$	273 $[2^+]$	781.4	781.2	3.5	1560.7
10.1	220–228	$1^+,2^+,3^+$	108 $[2^+]$	545.1	545.0	1.7	1088.1

[a]Amino acid residues are numbered according to the murine PrP sequence as in Westaway et al. 1987.
[b]Relative abundance = counts s^{-1} at the chromatographic peak maximum for the most abundant ion/charged state; the charge state [n+] is given in parentheses.
Xcorr indicates the quality of match of experimentally obtained spectra compared with software-predicted MS^2 peptides (SEQUEST) as represented by the cross-correlation score (see 'Analysis of tryptic peptides of PrP^{res} from murine TSEs by LC-MS^2).
MM_{avg}, average molecular mass; (-), insufficient data.

tryptic peptides (tryptic cleavage at C-terminus only) indicate that the probability of false-positive identification of murine or ovine tryptic PrP peptide would be of the order of 1% (Peng et al. 2003).

Target peptides were positively identified on the basis of: peptide mass, the SEQUEST match (Xcorr value) and the presence of associated doubly, triply and quadruply charged ions. Where target ions were not present at sufficiently high abundance to trigger the collection of MS^2 spectra, identification was also based on the proximity of the retention time ($\pm 10\%$) to that of the same peptide in the 301V strain (from which target peptides were generated in relatively high abundance), with the caveat that the Xcorr value for a given 301V peptide was >2.0.

The ion abundance of each peptide (counts s^{-1} at peak apex) was calculated using the value for the most abundant charged ion present. Values were compared (Table II) after normalizing to the abundance of the internal standard hydrolysis product bocVLK.

Results

TSE transmission to mice

All BSE and scrapie strains and field case isolates were successfully transmitted to appropriate mouse strains. The mean time to clinical disease varied with strain (Table I), as widely established. The lengthy incubation periods observed for the ovine scrapie (480 ± 40 days) and bovine BSE (483 ± 35 days) brain isolates are in keeping those expected at primary passage.

Western immunoblotting

Examination of PrP^{res} glycoform patterns from brain of TSE-inoculated mice demonstrated the characteristically lower molecular weight of unglycosylated PrP^{res} associated with BSE (301V and field-case BSE) compared with scrapie (22A, 87V, 79A, ME7 and field-case scrapie) infected animals (Figure 1). A similar shift was also observed for bands from positive control samples from BSE- and scrapie-infected bovine and ovine subjects. Visual comparison of banding patterns indicated differences in staining intensity in the order ME7 > 22A > 79A and 301V > 87V > field BSE and field scrapie.

Evaluation of variability of the tryptic peptide digestion process

The abundance of the tryptic product bocVLK enabled correction for process variability from the trypsin digestion stage onwards. The mean ($\pm$SD) response for bocVLK for a representative sample batch was 316.2 ± 101.4 ($n = 28$). Normalization of data against total protein in PrP^{res} preparations from individual brains was not feasible due to the limited quantities of material available.

Analysis of tryptic peptides of PrP^{res} from murine TSEs by LC-MS

The relative quantity and number of tryptic peptides generated from PrP^{res} varied appreciably with strain. The highest apparent abundance of PrP^{res} tryptic peptides was seen consistently for the 301V strain. This is illustrated by the typical LC-MS-extracted ion chromatograms shown for each strain (Figure 2). No significant quantity of peptides

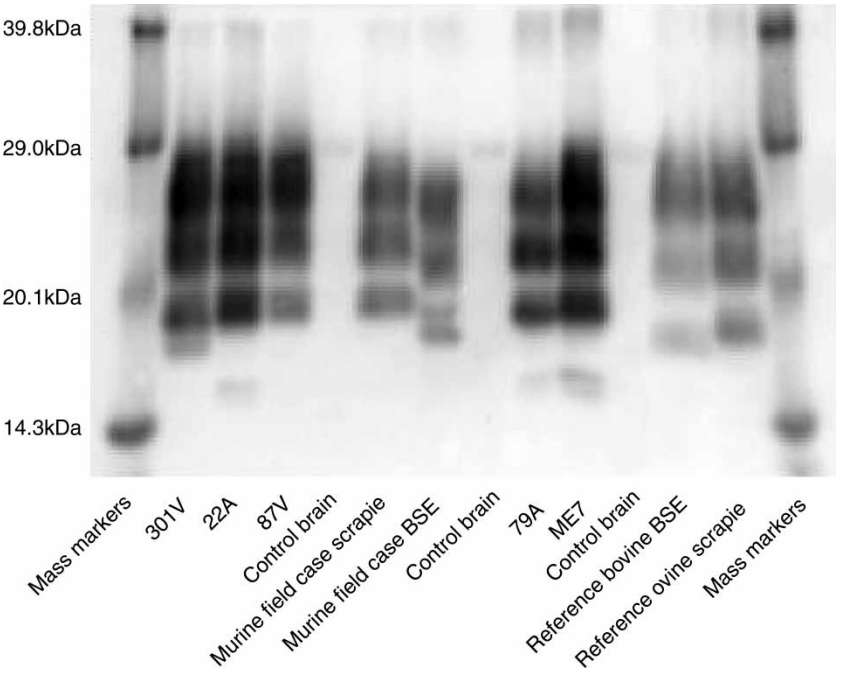

Figure 1. Western blot analysis of PrP^{res} of various mouse TSE strains, detected using 6H4 antibody.

was detected either in samples from field scrapie or BSE cases at first passage (low $[PrP^{Sc}]$ can be expected at first passage (Laurent 1998)) or from control mice. All peptides comprising amino acid residues between 73 and 105 are derived from PK N-truncated PrP^{Sc} and have C-termini at residue K^{105}. The other more C-terminal PrP peptides detected, can be assigned to tryptic proteolysis products only (cleavage adjacent to arginine or lysine, see Tables II and III). The possibility cannot be excluded, however, that some of these C-terminal peptides may be products of endogenous (e.g. proteasomal) proteolysis (Yedidia et al. 2001, Yadavalli et al. 2004), although this possibility is not considered further here.

Relative yields of peptides from 301V were greater (approximately fivefold) than from strains 87V and 79A and substantially higher ($\sim$50-fold) than from ME7 and 22A (data not shown). Correspondingly, appreciably more tryptic peptides (up to 20) were detected from 301V PrP^{res} (Table II) than from other strains, and peptides covered up to 70% of the PrP^{res} sequence.

Depending on the strain, between three and 14 peptides were detected from the N-terminal region of PrP^{res} revealing the relative complexity of the consensus PrP^{res}. All these peptides contained the C-terminal motif KPSKPK, which gives rise to unusual and distinctive triply and quadruply charged ions (up to 5^+ for some peptides), consistent with the presence of three lysine residues (Figure 3a), and a prominent series of MS^2 product ions which enabled sequence confirmation (Figure 3b).

Reference peptide standards were not available for calibration to enable full quantification, since requirements could not be established in advance. Accordingly, quantitative inferences may be made in comparing relative abundances (e.g. between strains) of the same peptides (Bondarenko et al. 2002), although comparisons between different N-terminal peptides are considered to be semiquantitative.

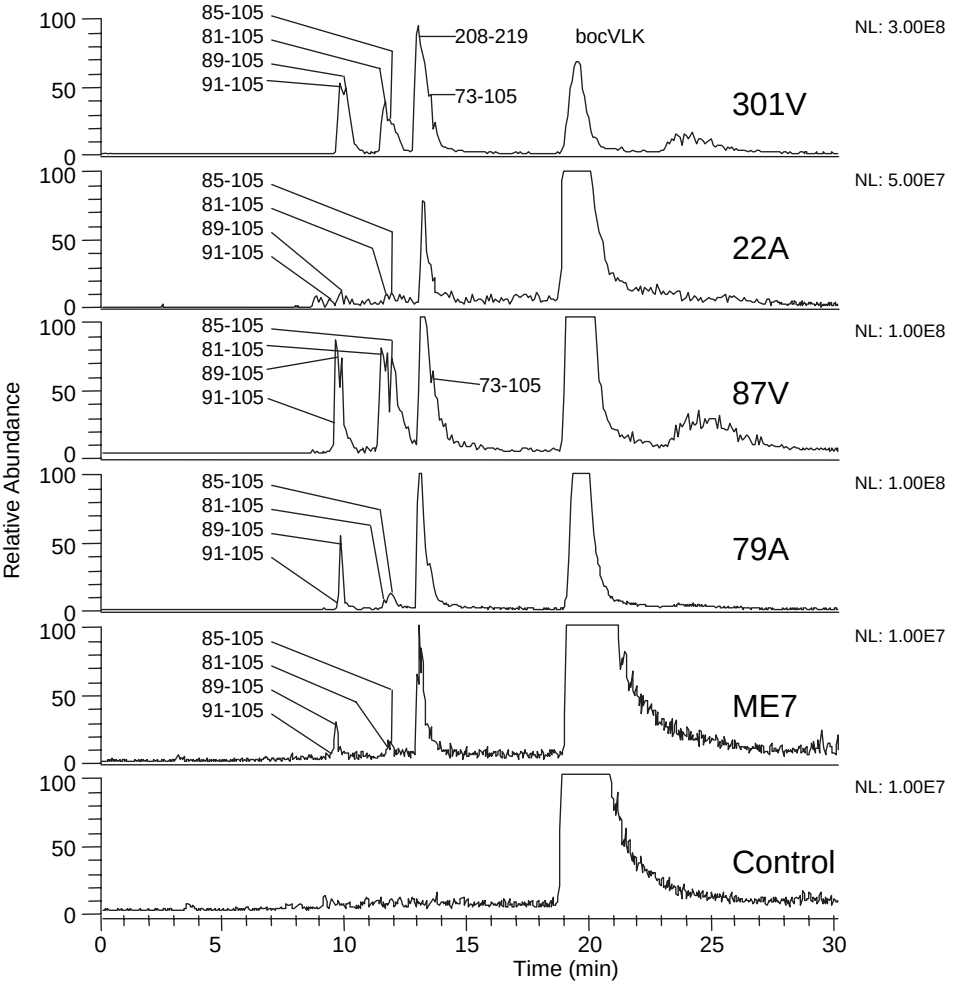

Figure 2. LC-MS-extracted ion chromatograms of tryptic peptides from mouse brain PrP^{res}: strains 301V, 22A, 79A, 87V and ME7 compared with control brain. The annotated ions correspond to selected peptides given in Table II, where their respective masses and charge states are indicated. Relative abundance values were normalized to the most intense PrP peptide associated peak. NL, normalized level.

Nevertheless, subsequent studies have demonstrated that MS detection efficiencies vary little between these peptides (maximally 50%; A. Gielbert & M. J. Sauer, unpublished results). Thus ratios of ion abundances of the major peptides provided a useful means of comparing the extent of variation of the PrP^{res} N-terminal sequence between strains. With the above in mind, it appears that tryptic peptide sequences 81–105, 89–105 and 91–105 were generated from 301V PrP^{res} in similar abundance (Tables II and III), with peptide 85–105 less so (PK cleavage at W^{80}, W^{88}, Q^{90} and H^{84}, respectively). Only these four peptides were detected consistently in PrP^{res} across the scrapie strains (Figure 4), reflecting overall lower concentrations compared with 301V. These cleavage points and the known murine PrP sequence enabled calculation of the molecular masses for the dominant PrP^{res} variants; for unglycosylated PrP^{res} (excluding GPI anchor) these approximated to 19.5, 18.7,

Table III. PrP^{res} N-terminal tryptic peptides and the main proteinase K (PK) cleavage sites for each mouse TSE strain.

PrP^{res} N-terminal tryptic peptide amino acid residue sequence[a]	TSE strain[b]
73 GQPHGGSW GQPHGGGWGQ GGGTHNQWNK PSKPK 105	$301V^3$, $87V^3$
81 GQPHGGGWGQ GGGTHNQWNK PSKPK 105	$301V^1$, $22A^2$,$79A^2$, $87V^1$, $ME7^1$
85 GGGWGQ GGGTHNQWNK PSKPK 105	$301V^2$, $22A^2$,$79A^2$, $87V^2$, $ME7^2$
89 GQ GGGTHNQWNK PSKPK 105	$301V^1$, $22A^1$,$79A^1$, $87V^1$, $ME7^1$
91 GGGTHNQWNK PSKPK 105	$301V^1$, $22A^2$,$79A^2$, $87V^3$, $ME7^2$

Full-length murine PrP[a]

1	MANLGYWLLA	LFVTMWTDVG	LCKKRPKPGG	WNTGGSRYPG	40
41	QGSPGGNRYP	PQGGTWGQPH	GGGWGQPHGG	SWGQPHGGSW	80
81	GQPHGGGWGQ	GGGTHNQWNK	PSKPKTNLKH	VAGAAAAGAV	120
121	VGGLGGYMLG	SAMSRPMIHF	GNDWEDRYYR	ENMYRYPNQV	160
161	YYRPVDQYSN	QNNFVHDCVN	ITIKQHTVTT	TTKGENFTET	200
201	DVKMMERVVE	QMCVTQYQKE	SQAYYDGRRS	SSTVLFSSPP	240
241	VILLISFLIF	LIVG 254			

[a]The primary sequence is as described (Westaway et al. 1987).

[b]Superscript numbers for each TSE strain indicate whether the N-terminal peptide represents a major, intermediary or minor (1, 2 and 3, respectively) PK cleavage product.

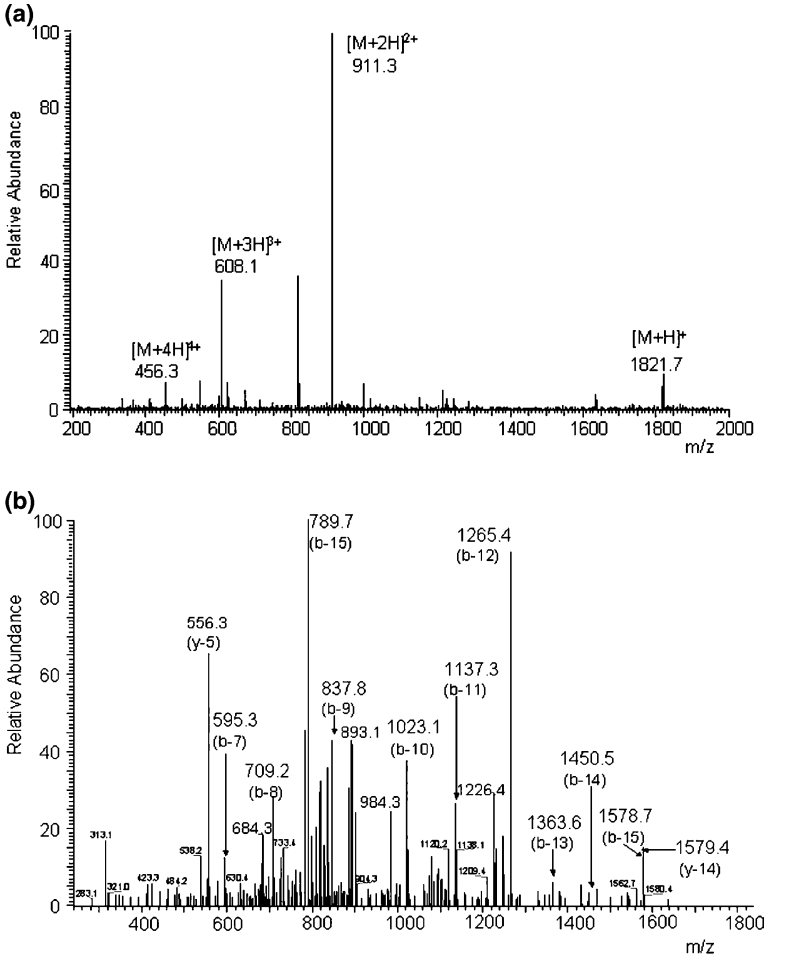

Figure 3. Nano LC-MS spectra of tryptic peptides; characteristics of N-terminal amino acid residues 89–105 from 301V P^{res}. (a) Peptide 89–105 (GQGGGTHNQWNKPSKPK) characteristically gave four charged states as indicated. (b) Sequence confirmation by MS^2; precursor ion ([M+2H]$^{2+}$, *m/z* 911.6) gave a distinctive series of singly charged b-ions (b-7 to b-15).

18.6 and 19.1 kDa, respectively, and 20.3 kDa for the longer peptide sequence 73–105 (cleavage at W^{72}). The latter was only detected consistently in strains 301V and 87V as a minor product (Figure 4).

Analysis of log-transformed abundance data (Figure 4) for these five peptides (73–105, 81–105, 85–105, 89–105 and 91–105) indicated a significantly higher content ($P \leq 0.01$; one-way analysis of variance (ANOVA) of log-transformed data) of peptide 91–105 from 301V PrP^{res} than from any scrapie strain. The content of peptide 89–105 in ME7 PrP^{res} digest was significantly less than in the other strains ($P \leq 0.01$).

Calculation of the ratios of relative abundance for all ten permutations of pairs of the five peptides (see Table III and Figure 4) within each strain, enabled between-strain comparisons of the relative proportions of each PK N-terminal truncation. Figure 5 summarizes ratios for the four peptide pairs which gave *p*-values <0.05 (Student's *t*-test assuming unequal variance was applied to accommodate heterogeneity between

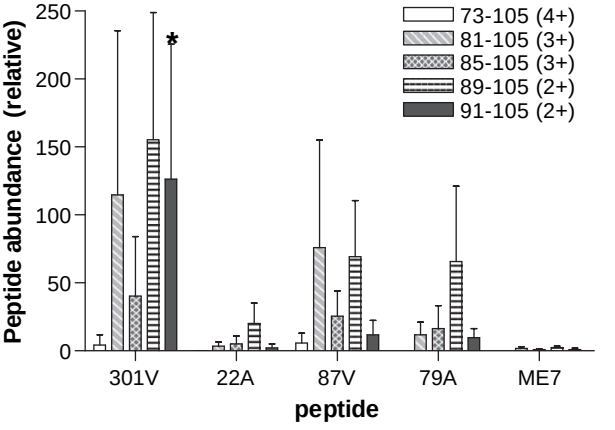

Figure 4. Relative abundance values (mean ± SD, $n = 4$) for the principal PrP^{res} N-terminal tryptic peptides from strains 301V, 22A, 87V, 79A and ME7. *The value for peptide 91–105 was significantly higher for 301V ($p < 0.01$; one-way analysis of variance following log transformation of data) than for other strains.

groups). Interestingly, for 301V, the abundance ratio of the shortest peptide 91–105 to peptide 89–105 was considerably greater than for all other strains (except ME7), and indeed for their combined means ($p \leq 0.01$). These data indicate (Figure 5) the possibility of discrimination between strains based on the abundance ratios of four (peptides 91–105:89–105, 91–105:85–105, 91–105:81–105 and 89–105:81–105) of the ten peptide pair permutations. Peptide ratios 91–105:89–105 and 91–105:85–105 for instance gave good discrimination between 301V and 22A, 79A and 87V. Similarly the ratios of 89–105:81–105 enabled discrimination between 301V and 79A and between 79A and 87V. As the abundance of peptides derived from ME7 were very low and presumably as a consequence, gave highly variable ratios, no distinction could be readily made between this and other strains. Thus although 301V could be readily distinguished from scrapie strains (except ME7), at this stage of test development the ability to readily distinguish between scrapie strains is more limited.

Discussion

Increased awareness of the diversity of TSE strains in humans, livestock and wildlife drives the need for higher-resolution means of molecular strain characterization to provide for better differential disease diagnosis and epidemiological investigation. The primary aim here was thus to evaluate the principle of MS-based N-TAAP, to establish the diversity of PrP^{res} within and between strains. Resolving PK cleavage points in this way, to the level of the individual amino acid, provides the potential to delineate molecular characteristics sufficiently to differentiate strains which would otherwise remain intractable by current biochemical techniques.

Data presented here provide strong support for this approach and indicate the extent of structural diversity within the N-terminal sequence of PrP^{res} of various mouse-passaged strains. This diversity is taken to reflect the extent to which strain-associated PrP^{Sc} conformation differentially limits sensitivity to PK digestion. Interestingly, whereas WB enables differentiation of certain strains from PrP glycoform migration patterns which are indicative of contrasting cleavage points, MS

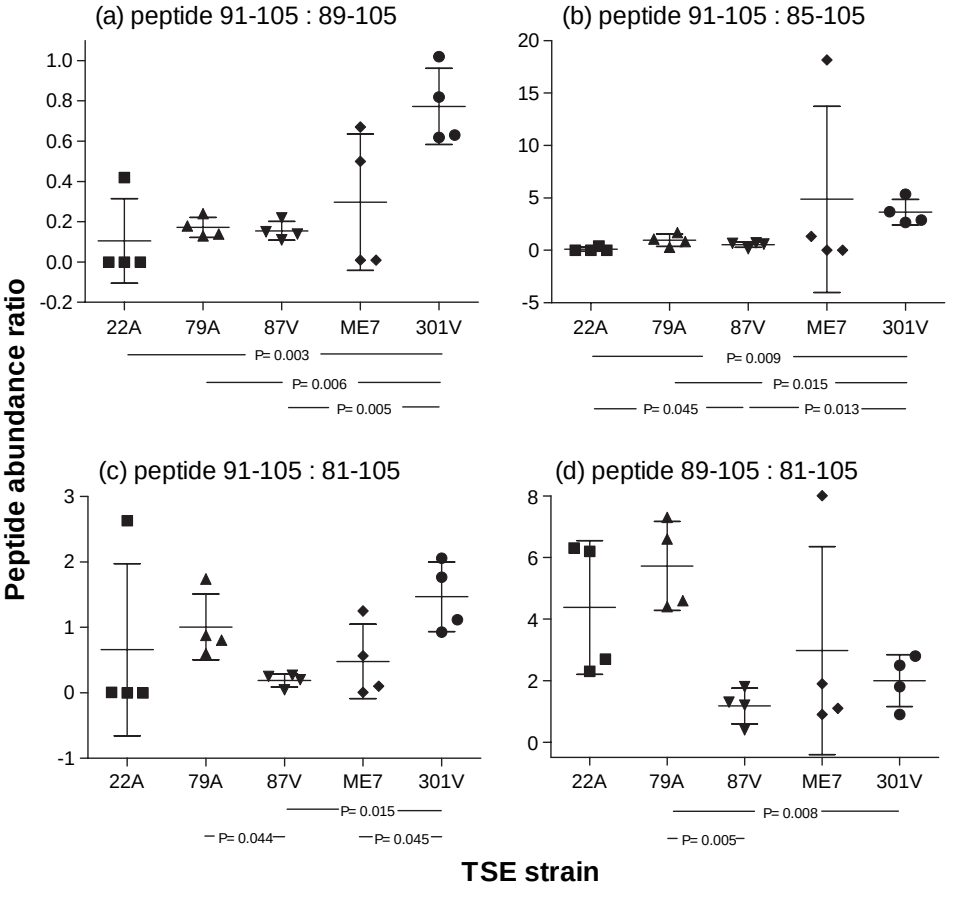

Figure 5. Peptide abundance ratios with potential to discriminate between 301V, 22A, 79A, 87V and ME7. Ratios of normalized ion abundance values were calculated for samples from individual animals ($n = 4$) and the mean $\pm$SD calculated for the ten permutations of pairs of the five peptides (as in Figure 4). Ratios that gave p-values < 0.05 are shown in (A)–(D). Pairs of strains where the ratios differed appreciably are denoted by the span of the horizontal lines beneath the x-axis and their associated p-values (Student's t-test assuming unequal variances) beneath the x-axes. The substantial variation of abundance values for ME7 samples reflects the low concentrations found, which were often at or near the detection limit.

analysis provides a different interpretation of the nature and extent of PK cleavage. Rather, MS points to numerous cleavage sites which overlap between strains, individual strains being distinguished by the relative proportions of the cleavage products.

It is well established that the production of PrP^{Sc} is a dynamic process which depends, during infection, on little understood complex physicochemical interactions involving interplay between infective agent and host PrP^{c} within the host biological milieu. This generates PrP^{Sc} with unique, strain-defined conformational boundaries. The present study uses PrP^{res} derived from whole brain to model these strain-related conformational strictures. A range of biological variables are known to influence the biochemical phenotype and diversity of PrP^{Sc} structure. Characteristic incubation periods are observed for different strains which can be modulated by the host PrP genotype (Bruce et al. 1991, Bruce 2003). Different strains give rise to distinctive

PrP^{Sc} deposition in the brain, reflected for instance in: unique cellular and regional distribution patterns (van Keulen et al. 1995, Ryder et al. 2001, Bruce 2003, Gonzalez et al. 2003, Khalili-Shirazi et al. 2005), morphological appearance (Ryder et al. 2001), cellular or extracellular location in full-length and truncated forms (Jeffrey et al. 2006), and degree of glycosylation and variability in susceptibility to PK digestion (Somerville et al. 1997, Stack et al. 2002, Thuring et al. 2004, Khalili-Shirazi et al. 2005, Yull et al. 2006). The underlying mechanisms involved are little understood, but variation in regional expression of conformers of PrP^{c} and their truncated and full-length glycoforms and isoforms may play a role (Beringue et al. 2003, Zanusso et al. 2004).

Achieving a level of sensitivity sufficient to characterize PrP^{res} in individual mouse brains required a number of analytical challenges to be met. Extraction of PrP^{res} from brain in sufficient quantity and purity required sequential application of both NaPTA precipitation and centrifugation through a sucrose cushion. Use of a capillary precolumn enabled high-volume sample loading, substantial enrichment of tryptic PrP^{res} peptides and elution in a minimum volume onto the analytical nano LC column. The latter approach maximized sensitivity of detection of eluted peptides by full-scan mode MS. Concurrent identification by MS^2 was achieved by data-dependent scanning based independently on both ion intensity and pre-selected *m/z* values.

Detection and identification of the major N-terminal peptides derived from individual mouse brains proved to be a practical proposition (Table III), although clearly, the sensitivity of the current method limited the number of less-abundant peptides detected for scrapie strains compared with BSE. Ratios of peptides abundances enabled comparison of variation between strains of the range of exact N-terminal cleavage points and their frequency of occurrence. The N-TAAP was appreciably simplified since only lysine residues are present for tryptic cleavage and it was evident that tryptic digestion at K^{100} and K^{103} was blocked (presence of C-proximal proline) (Keil 1992). Consequently the peptides from this region all C-terminated with the distinctive KPSKPK motif. The N-TAAP revealed 14 PK cleavage points (up to residue 105) for 301V, indicating substantial raggedness and, based on amino acid sequence, variation in the mass of PrP^{res} by up to 1769 Da (N terminus at G^{73} compared with G^{91}).

Peptide abundance data indicated that the major PrP^{res} variants N-terminated at G^{81}, G^{85}, G^{89} and G^{91} for all the strains (Table III). Significantly, the abundance ratio for residues 91–105:89–105 was higher for 301V compared with other strains, indicating a higher proportion of the most truncated PrP^{res} isoform (residues 91–231). This ratio approach thus enabled discrimination of 301V from all the scrapie strains and three of the four scrapie strains (87V from 79A and 22A) from each other, demonstrating the potential for differential diagnosis. Further preliminary studies have demonstrated wider application to characterization of sheep PrP^{res}. Using an identical approach to analyze a brain pool from scrapie-infected sheep, three N-terminal peptides ($G^{85–109}$, $G^{89–109}$, $G^{94–109}$) were found ($G^{96–109}$ not detected) indicating major cleavage points analogous to those found in mouse scrapie samples and similarly, giving a low-abundance ratio of $G^{96–109}$:$G^{94–109}$ ($\equiv G^{91–10}$:$G^{89–105}$ in mouse). The power of discrimination achieved thus far would nevertheless benefit from more sensitive and robust quantification, since overall variability of mean abundance values for each peptide and strain were relatively high (Figure 4). Ongoing

work indicates that these issues are far from insurmountable since both sensitivity and assay precision can be improved substantially by introduction of peptide standards for calibration and full quantitation by triple quadrupole MS in multiple selected reaction monitoring (mSRM) mode. This approach has enabled characterization and discrimination of various TSEs (classical scrapie, experimental BSE and scrapie isolates SSBP/1 and CH1641) by analysis of PrP^{res} from sheep brain stem (A. Gielbert & M. J. Sauer, unpublished results). Further improvements in robustness may be reasonably anticipated by incorporation of stable isotope-labelled internal standards for absolute quantification of key peptides.

The N-TAAP for 301V and scrapie strains largely reflected the WB pattern (Figure 1) particularly in relation to the proportion of lower-mass unglycosylated isoform seen for 301V (~18 kDa) compared with the scrapie strains (19–20 kDa). Since the N-TAAP assesses total PrP^{res}, the degree to which variation in the pattern of truncation is mirrored in each of the glycoforms remains an intriguing question, albeit one beyond the scope of these studies. This is an important detail, particularly since both the mobility (molecular mass) and relative proportions of individual glycoforms underpin characterization of strain diversity by WB (Stack et al. 2002, Head et al. 2004, Khalili-Shirazi et al. 2005, Yull et al. 2006). In the present study, individual WB glycoform bands were relatively diffuse, perhaps partially as a result of population of each zone by numerous unresolved truncated forms of PrP^{res}.

In recent studies (Hayashi et al. 2005) Edman sequencing enabled determination of N-terminal cleavage sites of PrP^{Sc} and differentiation between BSE and scrapie in mice. In keeping with findings here, distinct PK cleavage sites were observed for scrapie at G^{81}, G^{85} and G^{89}; however, these varied with the concentration of PK used; digestion at 25 μg PK g^{-1} brain gave a dominant cleavage point at G^{81} whereas 800 μg PK g^{-1} brain gave convergence to cleavage at G^{89} (Hayashi et al. 2005). The dominant PrP^{res} N-terminus from mouse-passaged BSE converged to N^{96}, irrespective of PK concentration, contrasting with the major cleavage point at G^{91} found here (Table III) to be the shortest sequence termination for the 301V BSE strain. Their studies provided an apparently more clear-cut discrimination between extracts of pooled brain samples from scrapie and BSE strains, both by sequencing and WB, than was observed here for individual brain samples from a range of strains, using tryptic digestion and peptide mapping. These differences may reflect the particular TSE isolates/strains studied, the PrP isolation method and differences in host mouse strain and specificity of antibody used for WB analysis. The MS approach detected a greater number of minor cleavage sites, however, in keeping with previous studies of ME7 PrP (Hope et al. 1988), and provided evidence for differences in dominant cleavage points between scrapie strains (Figure 4), indicative of greater potential for strain differentiation. It is likely that future optimization of the limited PK proteolysis process (e.g. PK concentration, pH, incubation time, etc.) will give rise to improvements in strain resolution since PK digestion conditions can clearly effect the position and extent of cleavage (Hayashi et al. 2005) or indeed give rise to complete digestion of PrP^{Sc} from certain strains (e.g. atypical scrapie; Langedijk et al. 2006). It remains to be clarified however, whether strain differentiation would best be met by limiting PK digestion, so as to generate the maximum diversity of N-terminal cleavage points, or by driving digestion closer to completion, so to provide one dominant cleavage point for each strain. Similarly, if the degree of N-truncation varies

between glycoforms, benefit may be gained from analysis of individual glycoforms and total de-glycosylated PrP^{res} (Nonno et al. 2003, Thuring et al. 2004).

A consistently higher abundance of N-terminal tryptic peptides was generated from PrP^{res} from BSE- compared with scrapie-inoculated mice (Figure 4), and this may have significance for strain differentiation. Whether this was truly due to higher concentrations of PrP^{Sc} in 301V brain, or whether the MS data reflected strain-related differences in, for instance, the selectivity of the PrP^{res} isolation procedure, remains to be fully established. This finding was intriguing, given the relatively low abundance of tryptic peptides observed for ME7 compared with both 301V and the other scrapie strains, and the contrast with WB data which indicated that bands for ME7 were at least as intense as those for all other strains. These apparently divergent outcomes probably more reflect differences in the selectivity of the distinct extraction procedure used for MS and WB. In this sense the N-TAAP approach stands independently as a method fit for this particular operational purpose. Particularly so, as the WB was used as a routine reference analysis rather than to provide a close methodological control. This was because the reduction and alkylation process used in the MS procedure was found, as elsewhere (Klingeborn et al. 2006), to reduce substantially PrP^{res} detection using 6H4 antibody.

The question of selectivity of different PrP^{res} extraction procedures is nevertheless an interesting and important consideration. Although, for a given species and PrP genotype, the sequence of the host PrP^{c} and PrP^{Sc} are understood to be identical, irrespective of the TSE agent strain, there is an array of complex interacting factors which impact on the physicochemical properties of host consensus PrP^{res}, and thus on the selectivity and efficiency of any extraction procedure. These include conformation, glycoform composition, aggregation state, regional anatomical location of the source material and the host PrP genotype. A range of extraction procedures have been developed for PrP in various laboratories over recent decades each having their own merits and selectivity profiles which have proven fit for particular purposes. For instance, relatively simple extraction processes using detergent solubilization, clarification, PK digestion and denaturation (as here for WB) have been widely used for differential glycoform analysis for strain classification (Stack et al. 2002, Thuring et al. 2004); as PrP is maintained in solution, losses are likely to be minimal and the content representative. NaPTA precipitation provides high recovery of PrP^{res} and a means of enrichment enabling sensitive detection in low-content samples, whether by WB or immunoassay (Safar et al. 1998, Collins et al. 2005, Gretzschel et al. 2006, Lipscomb et al. 2007). Sucrose cushion centrifugation provides for isolation of high-purity fibrils, facilitating studies of the nature of the infective agent and PrP^{Sc} aggregation state and enabling strain diagnosis and determination of PrP^{res} tissue distribution (Diringer et al. 1983, Diringer & Kimberlin 1983, Kascsak et al. 1986, Caughey et al. 1997, Buschmann & Groschup 2005).

For N-TAAP analysis it was necessary to combine NaPTA and sucrose cushion procedures to provide a level of both enrichment and purity appropriate to high-resolution determination of the range and relative quantities of PK cleavage products. As less aggregated forms of PrP^{Sc} have reduced likelihood of recovery in sucrose cushion centrifugation pellets, such preparations would clearly give rise to a different PrP^{Sc} selectivity profile than the detergent solubilization/clarification preparations used in the reference WB approach. To our knowledge, the relative proportions of different aggregation states present in PrP^{res} has not been extensively investigated in

relation to disease strain (but see Diringer et al. 1983, Diringer & Kimberlin 1983, Silveira et al. 2005); however, because of dependency on conformation, the strain is likely to be a major influence on the dynamics of aggregate formation in the host. This would provide a plausible explanation for the differences observed here between WB and the N-TAAP procedure. Use of a more sensitive and selective mSRM LC-MS/MS approach has since enabled comparison of NaPTA and sucrose for PrP^{res} isolation (A. Gielbert & M. J. Sauer, unpublished results) and provided a clear indication that PrP^{res} recovery is reduced appreciably by the sucrose gradient stage. The concentrations of peptide differed by 5–10-fold in the order: NaPTA alone > NaPTA + sucrose > sucrose alone. WB analysis indicated that despite differences in recovery, the relative intensity and migration of the glycoform bands was not noticeably changed between methods, or when compared with the reference method (Stack et al. 2002).

The present studies strongly support the potential of limited proteolytic digestion by PK and N-TAAP by tryptic digestion and LC-MS/MS to establish the extent of conformational diversity of PrP^{Sc} and to enable qualitative and quantitative comparisons to be made between TSEs, as a basis for strain differentiation. In particular, the prospect of application of N-TAAP to sheep presents the possibility of differentiation between BSE and scrapie and, more challengingly, between scrapie strains that remain largely intractable to biochemical approaches such as WB. The generic nature of the MS analysis precludes the need for generation of species or strain-specific biological reagents such as antibody panels and enables the exact location and extent of PK cleavage points to be established. Methodological refinement such as automation and the use of MALDI-TOF MS to increase sample throughput should further enhance the utility of MS-based N-TAAP compared with antibody-based approaches such as WB. Additionally, N-TAAP may prove to be of value in better defining strain-specific epitopes for future immunoassays. Finally, with the future development of isotope-dilution internal standardization mSRM-based MS, will come the capability for improved reproducibility and highly selective and sensitive fully qualitative and quantitative analysis of PrP^{res}. To date, this has not proved possible with antibody-based approaches due to lack of availability of appropriate means of calibration.

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