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Transmissible spongiform encephalopathies (TSEs) are characterised by the accumulation in the tissues of affected individuals of an abnormal form (PrPSc) of a protein naturally produced by the host, the cellular prion protein (PrPC). In sheep, susceptibility to TSEs is tightly controlled by polymorphism at positions 136 (A or V), 154 (R or H) and 171 (R or Q) of the Prnp gene encoding the prion protein (PrP). Quantification of PrP variants at positions 136, 154 and 171 can be achieved by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometric analysis of the respective peptides 114–139, 152–159 and 160–171 obtained after tryptic digestion of the PrP protein. In this study we quantified the tryptic peptide 114–139 containing the first polymorphic site. Quantification was either relative, between variants of this peptide, or absolute with respect to the C-terminally 18O-labelled peptide obtained by hydrolysing known amounts of recombinant protein with trypsin in H182O. After purification of PrPC and PrPSc from the brain of two heterozygous sheep carrying either the ARQ/VRQ or ARR/VRQ genotypes, the proportion of each variant was measured. In the ARQ/VRQ animal, while both variants were equally represented in the normal isoform, the VRQ variant was predominantly found in the abnormal PrP protein, suggesting dissimilar behaviour of the two variants in the pathological process. The situation was even more contrasted in the ARR/VRQ animal where PrPSc was solely composed of the VRQ variant. These two examples clearly illustrate the value of MALDI-TOF analysis, combined with appropriate immunopurification techniques, in seeking a precise understanding of the influence of PrP polymorphisms on TSE pathogenesis.

Transmissible spongiform encephalopathies (TSEs) form a group of fatal neurodegenerative diseases affecting several mammalian species including humans (Creutzfeldt-Jakob disease), cattle (bovine spongiform encephalopathy) and sheep (scrapie). A key event in TSEs is the accumulation in the central nervous system of an abnormal form of a protein naturally produced by the host, the prion protein, PrP. This abnormal form (called PrP^{Sc}, for scrapie PrP) often accumulates in the brain as amyloid plaques or deposit, of which it is the major component. PrP^{Sc} derives from the normal form (PrP^{C} for cellular PrP) through post-translational modifications, which induce a conformational change and confer to PrP^{Sc} a partial resistance to degradation by proteases as well as a marked insolubility in the presence of detergents, leading to the formation of large aggregates known as scrapie-associated fibrils (SAF).

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In sheep, the incidence of scrapie is strongly influenced by polymorphisms in the host gene that encodes PrP. In particular, the polymorphic codons 136, 154 and 171 have long been known to be associated with disease susceptibility and to influence the pathophysiology of the disease. The major mutation associated with susceptibility or resistance is located at codon 171,[1,2] so Q_{171} and H_{171} homozygous animals are the most susceptible to scrapie. Whereas maximum resistance to scrapie is reported in sheep carrying two $A_{136}R_{154}R_{171}$ (ARR) alleles, $V_{136}R_{154}Q_{171}$ (VRQ) homozygous animals show the greatest susceptibility.[2–5]

In heterozygous animals expressing different alleles of PrP, the contribution of each allele to the formation of PrP^{Sc} from the two forms of PrP^{C} is not known and there is a need for analytical methods to quantify each haplotype of PrP specifically.

In 1996, soluble amyloid-β protein was immunoprecipitated from cultured cell media and quantified by matrix-assisted laser desorption/ionisation time-of-flight mass

spectrometry (MALDI-TOF MS) relative to synthetic peptide internal standards.[6] More recently, cardiac myosin heavy chain isoform ratios were determined by MALDI-TOF MS after purification and in-gel digestion. The two tryptic peptides allowing discrimination of the isoforms differed only by an alanine-valine substitution and their ion-current ratio was directly proportional to the isoform ratio before trypsin digestion. As internal standard, a synthetic peptide was mutated to allow the simultaneous quantification of the two isoforms.[7] Alterman *et al*.[8] also used MALDI-TOF MS to quantify cytochrome P450 isozyme after in-gel or in-solution trypsin digestion, using a CYP2B2 isozyme-specific peptide as internal standard.

In this study, we combined immunoprecipitation, sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and in gel-digestion of sheep brain PrP treated or not with proteinase K (this treatment allows specific analysis of PrP^{Sc}), followed by MALDI-TOF MS analysis to quantify separately the VRQ allele with regard to the ARQ and ARR alleles of PrP. The two PrP 114–139 peptides used in the present study to discriminate the ARQ, ARR and VRQ variants differ by an alanine-valine substitution. As in Helmke's study,[7] we show that the ion currents generated by two such peptides are directly proportional to their concentration. The 114–139 peptide, which contains a high amount of hydrophobic residue, was difficult to synthesise and proved to be poorly soluble. We therefore chose as internal standard the ARQ variant of recombinant sheep PrP labelled with the stable isotope ^{18}O by digesting it with trypsin in $H_2^{18}O$.[9,10] Under these conditions, ARQ, ARR and VRQ variant proportions could be accurately determined on 100 ng of total PrP per band of SDS-PAGE.

EXPERIMENTAL

Reagents

Monoclonal antibody Sha-31 was raised in PrP 0/0 mice by immunising with a preparation of native SAF obtained from a hamster brain infected with the 263K strain. It binds the 145–152 sequence of PrP (YEDRYYRE) and recognises both native PrP^{C} and denatured PrP (both from PrP^{C} and PrP^{Sc}), but fails to bind native PrP^{Sc}.

Recombinant PrP production

The ARQ variant cDNA was inserted into the pET22b vector (Novagen) (kind gift from H. Rezaei, INRA VIM, Jouy en Josas, France) and was mutagenised to the VRQ variant using the Quick Change mutagenesis kit (Stratagene).

For the expression, 400 mL of Luria Broth in a 2-L Erlenmeyer flask were seeded with 8 mL of an overnight preculture and grown for 2 h until the optical density (OD) reached 0.6. Expression was induced with 50 μM isopropyl-β-D-thiogalactoside (IPTG) for 5 h. The culture was centrifuged and the pellet resuspended in 20 mL of a 100 mM pH 7.4 potassium phosphate buffer and sonicated in 2×10 mL for 1 min.

After centrifugation the pellet was resuspended in 10 mL of Qiagen buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjusted to pH 8) and rotated for 1 h at room temperature. After centrifugation, the supernatant was mixed with 1.5 mL of decanted fast-flow Ni-NTA resin (Qiagen) and rotated for a further 1 h at room temperature. The slurry was then poured into a Bio-Rad Econo column (16×100 mm) and connected to a Bio-Rad Biologic LP chromatography system. The resin was washed at 6.5 mL/min with Qiagen buffer B until the 280 nm OD reached a baseline, and then washed at the same flow rate with a pH 7 adjusted buffer B. Elution was performed with Qiagen buffer C (pH 6.3 adjusted buffer B) at 3 mL/min. Pooled fractions were dialysed overnight against one 5 L change of 50 mM pH 4 sodium acetate buffer followed by an overnight 5 L bath of water. Water dialysed protein was stored frozen at −20°C and a fraction was lyophilised.

In-solution determination of recombinant ARQ/VRQ PrP ratio in artificial mixture

Recombinant ARQ and VRQ variants were mixed in the following ratios: ARQ/VRQ, 90:10, 88:12, 85:15, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 15:85, 12:88, 10:90 at three total final PrP concentrations: 800 μg/mL, 80 μg/mL and 8 μg/mL, in water. Triplicates of 20 μL of each preparation were mixed with 20 μL of 40 μg/mL modified porcine trypsin (Promega) in 50 mM ammonium bicarbonate pH 8 buffer and digested for 30 min at 50°C with vigorous mixing.[11] A volume of 1 μL of digest was mixed with 10 μL of CHCA matrix (10 mg/mL alpha-cyanohydroxy-cinnamic acid in 50% acetonitrile and 0.3% trifluoroacetic acid in water, Sigma, non-recrystallised) for the highest concentrations and 5 μL for the two lowest ones in Eppendorf tubes and 1 μL was spotted on a stainless steel MALDI plate. After drying, spots were directly analysed for the two highest concentrations and washed for the lowest one. To wash the spot, 10 μL of 0.1% TFA in water were applied to the spot, which was blown with pressurised air after about 30 s. Analysis was performed with a Voyager-DE STR (Applied Biosystems) MALDI-TOF mass spectrometer using the following instrument settings: positive mode, reflector, delayed extraction 140 ns, accelerating voltage 20000 V, grid voltage 66%, mirror voltage ratio 1.12, about 500 laser shots.

In-solution absolute quantification of ARQ and VRQ PrP variants

Recombinant sheep PrP ARQ and VRQ were diluted to 0.5 mg/mL (21.7 μM) in water, and were mixed in a total volume of 200 μL in the following ratios: ARQ/VRQ, 100:0, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 0:100. Duplicates of 20 μL of each mixture were digested with 20 μL of 40 μg/mL (1.7 μM) modified porcine trypsin (Promega) in 50 mM ammonium bicarbonate pH 8 for 30 min at 50°C with vigorous mixing.

For the preparation of the $H_2^{18}O$ internal standard, lyophilised ARQ was resuspended in $H_2^{18}O$ at 0.38 mg/mL (16.5 μM) and 20 μL were transferred to an Eppendorf tube containing 400 ng of dried trypsin and 1 μmol of dried 1 M ammonium bicarbonate pH 8 buffer and incubated as above. After digestion, the tracer was diluted with 980 μL of CHCA matrix at a final concentration of 0.33 μM. Volumes of 2 μL of each ARQ/VRQ ratio were then added to 40 μL of the matrix diluted tracer and analysed as described above.

'In-gel' determination of ARQ/VRQ ratio and ARQ or VRQ absolute concentrations

Gel bands were excised from the SDS-PAGE gel and cut into 1 mm^3 fragments. They were washed successively for 10 min with water and 100% acetonitrile. Acetonitrile was removed and the fragments were dried in a vacuum concentrator. They were then digested with 50 μL of 20 ng/mL porcine trypsin (Promega) for 30 min at 50°C, and were either analysed immediately or the following day. When the samples were left overnight at 4°C, the signal was stronger than when the samples were analysed immediately. We believe that this simply reflects the fact the time overnight at 4°C allows a greater diffusion of the peptides out of the gel. The samples were either analysed directly by spotting 1 μL of digest on the MALDI plate and adding 1 μL of CHCA matrix, or after mixing five times with the $H_2^{18}O$-digested recombinant PrP. The same procedure was used to analyse PrP^{Sc} from VRQ/ARR animals.

PrP^C and PrP^{Sc} purification from sheep brain extracts

Brains from healthy ARQ/VRQ sheep were obtained from a scrapie-free flock (VLA Weybridge), while sheep clinically affected with scrapie were obtained from a naturally scrapie-affected flock (Langlade flock, Castanet Tolosan, France). After culling, brains were collected and snap frozen in liquid nitrogen and stored −80°C before further processing. Healthy and affected animals were age-matched (30 months old) and genotype was checked by sequencing of the full Prnp open-reading frame (ORF) using brain DNA extract and no other variations of the PrP gene potentially interfering with the analysis was found.

Brain tissues were homogenised (20% w/v) in 5% glucose using a Ribolyser (Bio-Rad). PrP^C and PrP^{Sc} were purified from brain extracts obtained from the brain homogenate using a mixture of detergents. Under these conditions, a brain extract from an infected animal contains both PrP^C, as a monomer trapped in micelles, and PrP^{Sc}, which is present mainly as large aggregates known as scrapie-associated fibrils (SAFs).

PrP^C was immunopurified as follows: the 20% brain homogenate was diluted twofold in 5% glucose and 6 mL of the corresponding 10% brain homogenate were diluted twofold with 2× extraction buffer (20 mM Tris pH 7.4, 20 mM EDTA, 0.2 M NaCl, 2% DOC and 1% NP40), incubated at 37°C for 10 min before clarification by centrifugation (500 *g* for 2 min). Recovered supernatant was diluted fivefold in extraction buffer and reacted overnight at 4°C with mAb Sha31 covalently coupled to Sepharose 4B gel beads (equivalent to 150 μL of wet gel). Under these conditions, with both infected and uninfected brain extracts, only PrP^C is immunopurified because the monoclonal antibody Sha-31 does not bind to aggregated PrP^{Sc}.

PrP^{Sc} was purified in two steps, first by preparation of SAFs using the Bio-Rad TeSeE purification kit and second by immunoprecipitation of denatured PrP^{Sc}. Briefly, SAFs were purified from 6 × 500 μL of 20% brain homogenate using the TeSeE purification kit (Bio-Rad), which includes extraction of PrP with a mixture of detergent, a proteinase K (PK) treatment to eliminate PrP^C and a centrifugation step to concentrate aggregated PrP^{Sc}.[12] The six corresponding pellets were denatured and dissolved in C1 buffer (a mixture of detergent and chaotropic agents) by treatment at 100°C for 10 min. They were then diluted tenfold in extraction buffer and reacted at room temperature for 4 h with 300 μL of Sha31 covalently coupled to Sepharose 4B. Under these conditions, PrP^{Sc} is selectively purified because PrP^C has been destroyed by the PK treatment and denatured PrP^{Sc} is actually recognised by the monoclonal antibody SHa-31.

After three washes with phosphate-buffered saline (PBS) containing 1% Tween-20, PrP was removed from the affinity gel by heating for 5 min at 100°C with 50 μL of Laemmli buffer (62.5 mM Tris/HCl pH 6.8; 2% SDS; 10% glycerol; 0.01% bromophenol blue). Samples were then fractionated by SDS-PAGE (12% acrylamide) as described earlier.[13] After electrophoresis, proteins were stained with Coomassie blue.

Immunoprecipitation efficiency was determined by measuring PrP^C and PrP^{Sc} in the supernatant using an enzyme-linked immunosorbent assay (ELISA) as previously described.[13] In all cases, more than 95% of the PrP was precipitated (results not shown).

RESULTS

Peptide mass fingerprinting of recombinant sheep PrP

MALDI analysis of in-solution (40 μL) hydrolysis of a 50% mixture of 80 ng of each ARQ and VRQ variants with 800 ng of porcine trypsin (ten times more than PrP) is shown in Fig. 1. Peptides identified using Protein Prospector software[14] with the enzyme Trypsin-Pro option (trypsin allowed to cut before a proline residue) are shown in Table 1. These data show that several 'basic-residue'-proline (K/R-P) bonds are cut. Although in Fig. 1 a hydrolysis temperature of 50°C was used, the same pattern of peptides was obtained with hydrolysis at 37°C. Trypsin usually cleaves proteins after lysine (K) and arginine (R) residues and is usually unable to hydrolyse a K/R-proline bond, but can sometimes do so, depending on the tertiary structure containing the K/R-proline bond. Sheep prion contains six K/R-P bonds at positions 26, 29, 104, 107, 139, 167. The first four are located in the octa-repeat regions: peptides MH+ 1045.47 (invisible on the figure because considered as an isotope of the 1044.46 by the software) and MH+ 1426.72 both result from cuts at positions 26 and 29. Serendipitously, the R-P bond at position 139 was also cleaved by trypsin and resulted in the generation of peptides MH+ 2331.16 (114–139^{A136}, abbreviated A136), 2359.19 (114–139^{V136}, abbreviated V136), and 1475.68 (140–151). This was beneficial to our study since otherwise the polymorphic site at position 136 would have been included in peptides of MH+ 3787.84 and 3815.88. Peptides of such a high molecular weight are generally poorly desorbed and give very low ion currents with poor resolution. As previously described, peptides containing a C-terminal arginine gave the strongest ion currents.[15] The expected peptide masses allowing discrimination of the different sheep PrP variants are shown in Table 2.

In-solution ARQ-VRQ ratio curves

Concentrations of recombinant PrP were determined by UV absorption. Attempts to use recombinant sheep PrP purified

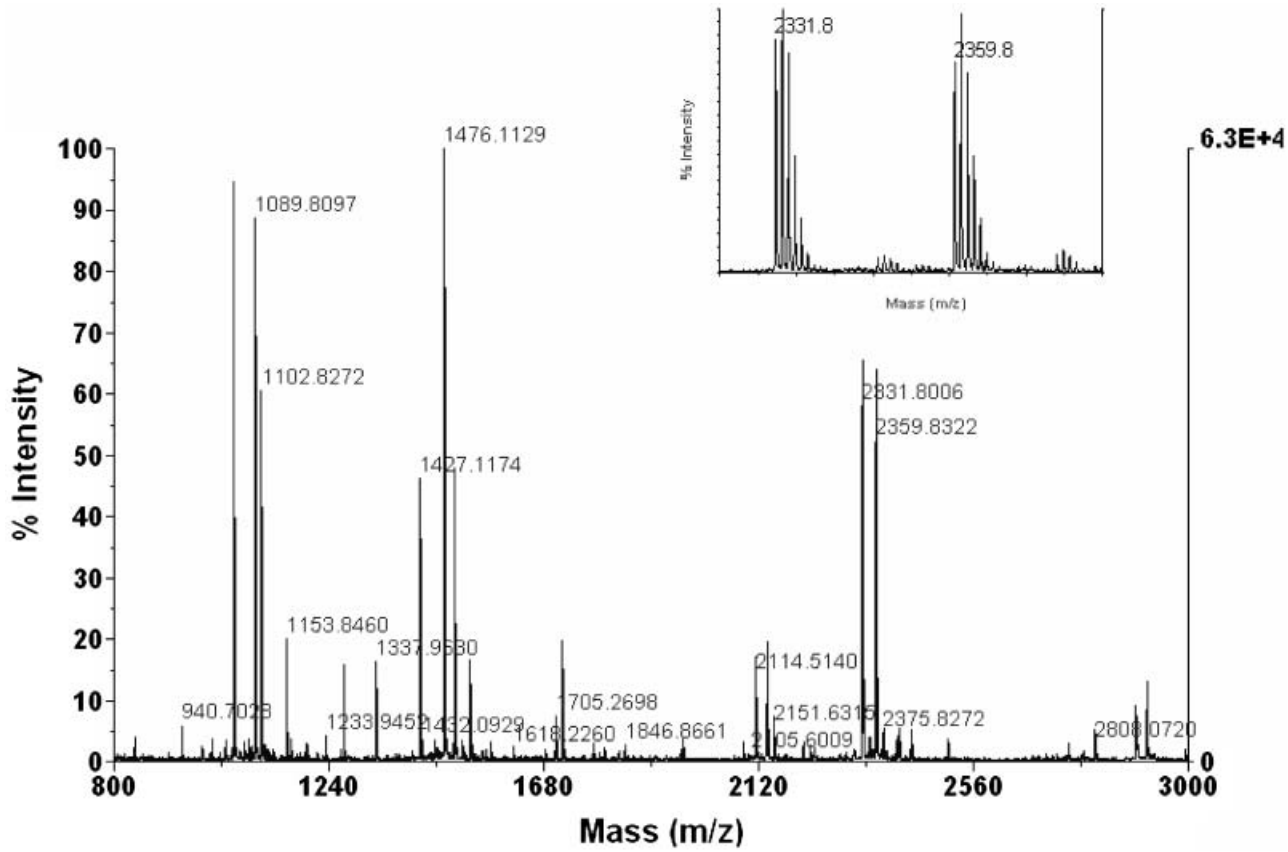

Figure 1. Peptide mass fingerprinting of the tryptic digest of a 50% mixture of ARQ and VRQ recombinant sheep PrP variants. Each variant (80 ng) and 800 ng of porcine trypsin in 40 μL of 25 mM ammonium bicarbonate pH 8 buffer were hydrolysed for 30 min at 50°C. The digest was diluted five times in CHCA matrix before analysis by MALDI-TOF MS. Trypsin autolysis peaks are at MH+ 842.5035 and 2211.1046 and the latter was used to calibrate the spectrum. The inset shows a magnification of the 2320–2390 *m/z* region. In this example, the peaks generated by oxidation of the methionine residue in position 132 of the two haplotypes are clearly visible (MH+ 2350.1478 and 2375.1945 for ARQ and VRQ, respectively).

according to Rezaei *et al*.[16] failed to give a good correlation between PrP variant ratio and peptide A136 and V136 ratios. In this method, PrP is bound in urea to an Immobilized metal affinity chromatography (IMAC) resin through its octa-repeats domain, refolded on the column and eluted with as high as 1 M imidazole. It is then subjected to a desalting G25 column purification to remove imidazole. It seems very likely that some imidazole remained after the G25 chromatography and that it biased the UV absorption measurement of PrP preparations. We therefore used another purification scheme

Table 1. PrP peptides identified after submission of the masses obtained in Fig. 1 to the Protein Prospector software.[14] In fact, peptides of MH+ 2359.1992 and 2375.1945 were not identified by the software because only the ARQ variant was included in the database, but they are listed in this table for the sake of clarity

m/z submitted	MH^+ matched	Delta ppm	Modifications	Start	End	Missed cleavages	Sequence
1044.4674	1044.4745	−6.8		224	231	0	(R) ESQAYYQR (G)
1045.4728	1045.4810	−7.8		30	40	0	(K) PGGGWNTGGSR (Y)
1089.4956	1089.5072	−11		41	51	0	(R) YPGQGSPGGNR (Y)
1102.5269	1102.5316	−4.3		160	167	0	(R) YPNQVYYR (P)
1153.5353	1153.5372	−1.6		198	207	0	(K) GENFTETDIK (I)
1270.6194	1270.6287	−7.3		28	40	1	(R) PKPGGGWNTGGSR (Y)
1426.7250	1426.7298	−3.4		27	40	2	(K) RPKPGGGWNTGGSR (Y)
1475.6860	1475.6914	−3.6		140	151	0	(R) PLIHFGNDYEDR (Y)
1497.7129	1497.7188	−4.0		212	223	0	(R) VVEQMCITQYQR (E)
2151.0310	2151.0564	−12		189	207	1	(K) QHTVTTTTKGENFTETDIK (I)
2331.1628	2331.1696	−2.9		114	139	0	(K) HVAGAAAAGAVVGGLGGYMLGSAMSR (P)
2347.1480	2347.1645	−7.1	1 Met-ox	114	139	0	(K) HVAGAAAAGAVVGGLGGYMLGSAMSR(P)
2359.1992				114	139	0	(K) HVAGAAAAGAVVGGLGGYMLGSVMSR(P)
2375.1945			1 Met-ox	114	139	0	(K) HVAGAAAAGAVVGGLGGYMLGSVMSR(P)

Table 2. Masses of the PrP peptides allowing discrimination of some of the sheep PrP variants. In this study we worked only on samples containing either the ARQ, ARR or VRQ variant of the PrP (recombinant or natural protein)

PrP variant	Peptide 114–139 MH+	Peptide 152–159 MH+	Peptide 160–171 MH+
ARQ	2331.17	—	—
AHQ	2331.17	1175.49	—
ARR	2331.17	—	1569.78
VRQ	2359.20	—	—

which avoided the use of imidazole. Recombinant PrP was still bound in urea through its octa-repeats domain to an IMAC resin but it was eluted still in urea by lowering the pH from 7 in the washing step to 6.3 in the elution step. At this pH proteins are usually considered to be bound through a 6xHis tag. However, the PrP octa-repeats domain probably has less affinity for Ni^{2+} ions than for a 6xHis tag. After elution, urea was removed by extensive dialysis first against a sodium acetate pH 4 buffer followed by water. PrP was stored at 800 μg/mL in water and was perfectly stable to freeze and thaw cycles.

Recombinant ARQ and VRQ variant ratio curves at three different total PrP concentrations are shown in Fig. 2. Each variant peptide was quantified by summing the total area of its isotope cluster. We found that this method gave better curves than simply using the height or the area of the first isotope. Baseline spectra were corrected with the advanced baseline correction (parameters: 150, 0, 0) of the Data Explorer software (Applied Biosystems). Good linearity was observed at the three total concentrations assayed, showing that the method can accurately determine the ratio between ARQ and VRQ in these synthetic mixtures.

Use of in-solution-$H_2^{18}O$ trypsin-digested recombinant PrP as an internal standard

To quantify the total amount of PrP recovered, we had to use an internal standard. The ARQ variant of recombinant sheep PrP was independently trypsin digested in $H_2^{18}O$ and then mixed with the sample. In the resulting isotopic cluster containing a mixture of $H_2^{16}O$ and $H_2^{18}O$ peptide, the proportion of each label was determined according to Bantscheff *et al*.[17] The first peak of the cluster contained only $H_2^{16}O$ peptide and was used to determine the proportion of $H_2^{16}O$ peptide of the following peaks. This proportion was calculated using theoretical isotopic ratios given by the GPMAW software. The concentration of the PrP dissolved in $H_2^{18}O$ being known by measurement of its absorbance, the concentration of $H_2^{16}O$ peptides could be determined, and are plotted against the concentrations in the ARQ-VRQ mixtures in Fig. 3. It can be seen that the linear regression curves drawn from the results are almost identical to the theoretical ones.

Comparison of in-solution and in-gel digestion of recombinant sheep PrP

Different amounts of mixtures of the ARQ and VRQ variants in the proportion 1/3 were digested either in-solution or in-gel in a 50 μL volume (see Experimental section). An amount of 30 ng of total protein was the limit at which both variant proportions could be accurately determined in-solution, compared with 100 ng total protein in-gel (data not shown). Quantification was performed relative to $H_2^{18}O$-digested ARQ variant (Table 3). These data clearly showed that two-thirds of the protein was lost in-gel under the conditions we used. It is worth noting, however, that the proportion between the two variants was kept in in-gel samples with respect to in-solution samples, and is very close to the theoretical proportion of the mixture.

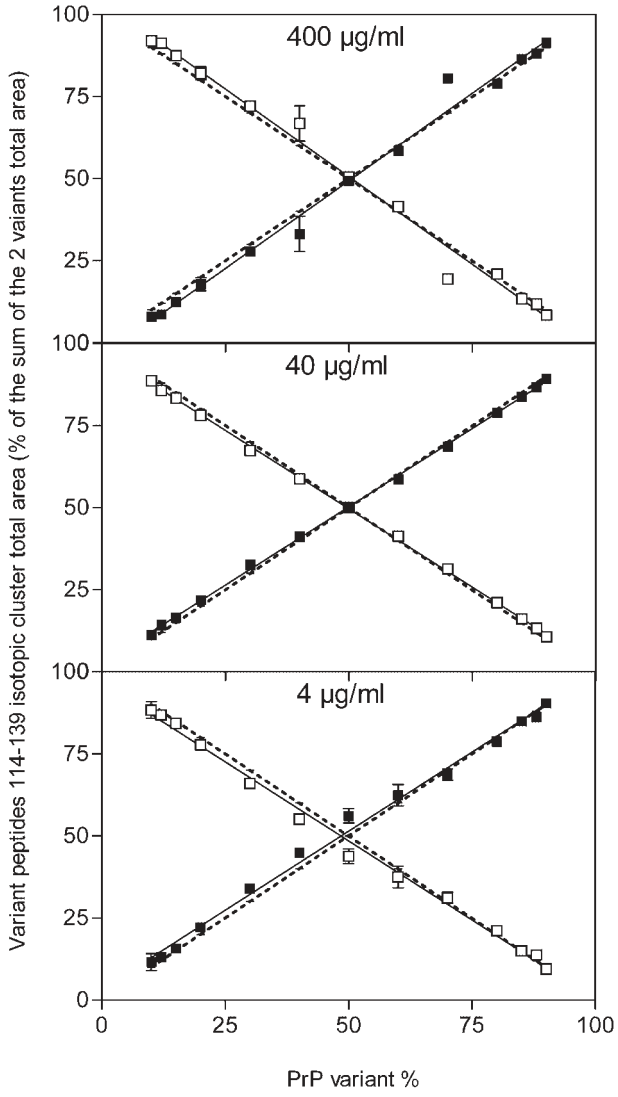

Figure 2. ARQ-VRQ ratio curves. PrP variants were mixed in various proportions and digested with 20 μg/mL porcine trypsin for 30 min at 50°C at the total final concentration indicated in each panel. The percent area of each variant peptide 114–139 determined by MALDI-TOF was plotted against the percentage of each digested variant PrP. Filled squares: ARQ variant; open squares: VRQ variant; dotted line: theoretical line. Each point is the mean ± standard error of the mean (SEM) of three experiments.

Analysis of ARQ/VRQ and ARR/VRQ sheep brain extracts

In PrP^C preparations obtained from both infected and uninfected ARQ/VRQ sheep (see Experimental section),

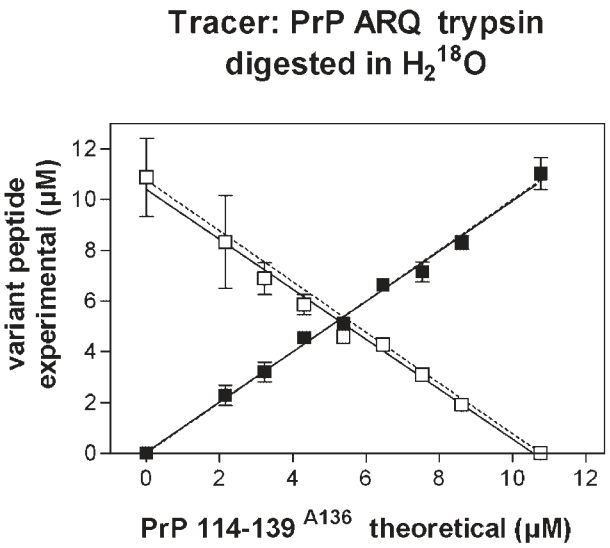

Figure 3. Use of 'in-solution' $H_2^{18}O$ trypsin-digested recombinant sheep ARQ PrP variant as an internal standard. ARQ and VRQ variants at different ratios and at 500 µg/mL total concentration were trypsin-digested in $H_2^{16}O$. The ARQ variant was independently trypsin-digested in $H_2^{18}O$ diluted 50 times with CHCA matrix in which the $H_2^{16}O$ digests were in turn diluted 20-fold. The proportion of $H_2^{16}O$-labelled peptide was calculated in each mixed label isotopic cluster and their concentration deduced. Filled squares: ARQ variant; open squares: VRQ variant; dotted line: theoretical line. Each point is the mean ± SEM of three experiments.

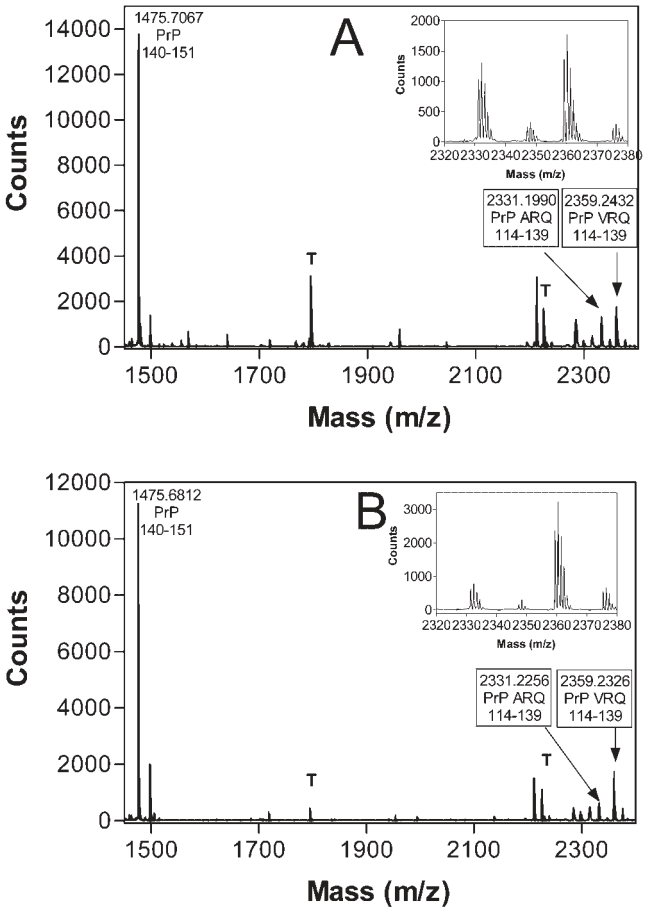

Figure 4. MALDI-TOF mass spectra in positive reflectron mode of the in-gel tryptic digests of immunoprecipitated PrP^C (A) and PrP^{Sc} (B) from an ARQ/VRQ sheep brain homogenate. One 1 mm slice among the nine spanning the PrP region (see Fig. 5) is represented. The same homogenate from a scrapie-infected brain was: (i) not treated with PK and not denatured (A), (ii) treated with PK and denatured (B) before immunoprecipitation with mAb Sha-31. Insets are the isotopic resolution of peptide 114–139 peaks, which contain the polymorphism site A136 or V136. Masses were calibrated with trypsin auto-digestion peptides 842.51 Da and 2211.1046 Da. Peaks labelled T result from trypsin auto-digestion.

A136 and V136 variants were observed in equivalent amount (see Figs. 4(A) and 5(A) for an infected brain). In contrast, in PrP^{Sc} preparations obtained from scrapie-affected ARQ/VRQ sheep, if both variants were observed the A136-V136 ratio was equal to 1/3 in all investigated samples (see Figs. 4(B) and 5(B)). This result clearly indicates a preferential accumulation of the VRQ variant in PrP^{Sc}.

Immunopurified PrP is composed of a heterogeneous population containing the different glycotypes of the protein (typically diglycosylated, monoglycosylated and unglycosylated PrP). In addition, the PK treatment is known to introduce a heterogeneity in the N-terminal part of the protein[18–20] so that, in SDS-PAGE, the apparent molecular mass of PrP varies between 30 and 40 kDa. In the Coomassie blue-stained gel patterns (Fig. 5), the gel region spanning the PrP protein was cut into nine slices before analysis of the A136-V136 tryptic peptides using MALDI-TOF (see Fig. 5). In the PrP^C preparation, equivalent amounts of A136 and V136 peptides were recorded whatever the band analysed, while in the PrP^{Sc} preparation, an A136-V136 ratio close to one-third was systematically observed. This clearly shows that our MALDI analysis is not influenced by the heterogeneity of the PrP preparation. This makes sense since the 114–139 peptide contains no glycosylation site and is not affected by the PK treatment.

Table 3. Comparison of in-solution and in-gel tryptic digests of an ARQ-VRQ 1/3 mix. The same amounts of a 1/3 mix of recombinant PrP ARQ and PrP VRQ at three total concentrations were digested either in-solution or in-gel with 20 µg/mL porcine trypsin in a 50 µL volume as described in the Experimental section. These digests were diluted five times in CHCA matrix containing 1 µg/mL in-$H_2^{18}O$ tryptic digest of the ARQ variant. Calculations were performed on the total area of the isotopic clusters. In parentheses: VRQ-ARQ ratio. The experiment was conducted once

		Quantification vs. $H_2^{18}O$-digested ARQ		
In-solution digest	ARQ (ng)	112	30	9
	VRQ (ng)	328 (2.9)	88 (2.9)	28 (3.1)
	Total (ng)	*440*	*118*	*37*
In-gel digest	ARQ (ng)	39	11	3.8
	VRQ (ng)	111(2.8)	32 (2.9)	9.8 (2.6)
	Total (ng)	*140*	*43*	*13.6*

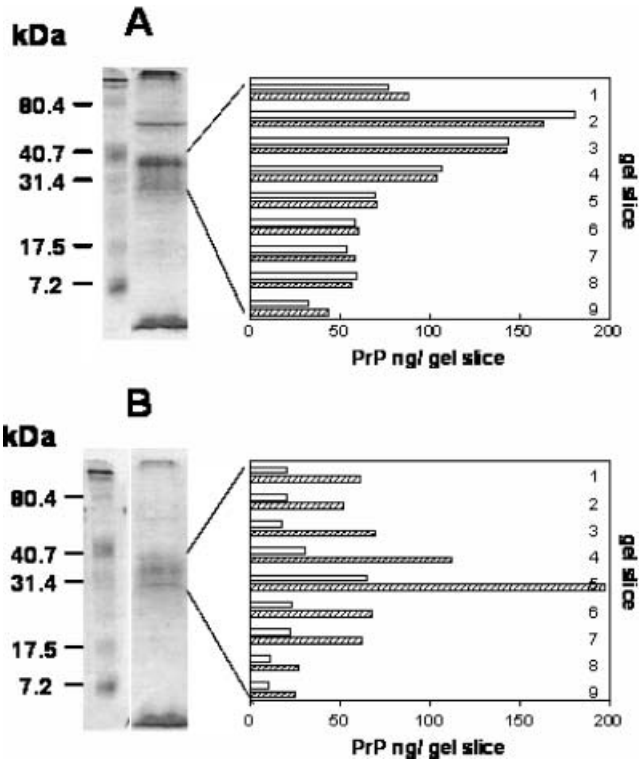

Figure 5. Quantification of each PrP variant in an ARQ/VRQ ovine brain PrP^{C} (A) and PrP^{Sc} (B). The amount of each variant of peptide 114–139 was quantified in the nine slices spanning the immunoprecipitated PrP isoforms separated by SDS-PAGE and stained with Coomassie brilliant blue. The same homogenate from a scrapie-infected brain was either not treated with PK and not denatured (A) or treated with PK and denatured (B) before immunoprecipitation with mAb Sha-31. A track of kaleidoscope prestained standards (Bio-Rad) was added on the left of the PrP tracks. Open bars: ARQ variant. Hatched bars: VRQ variant.

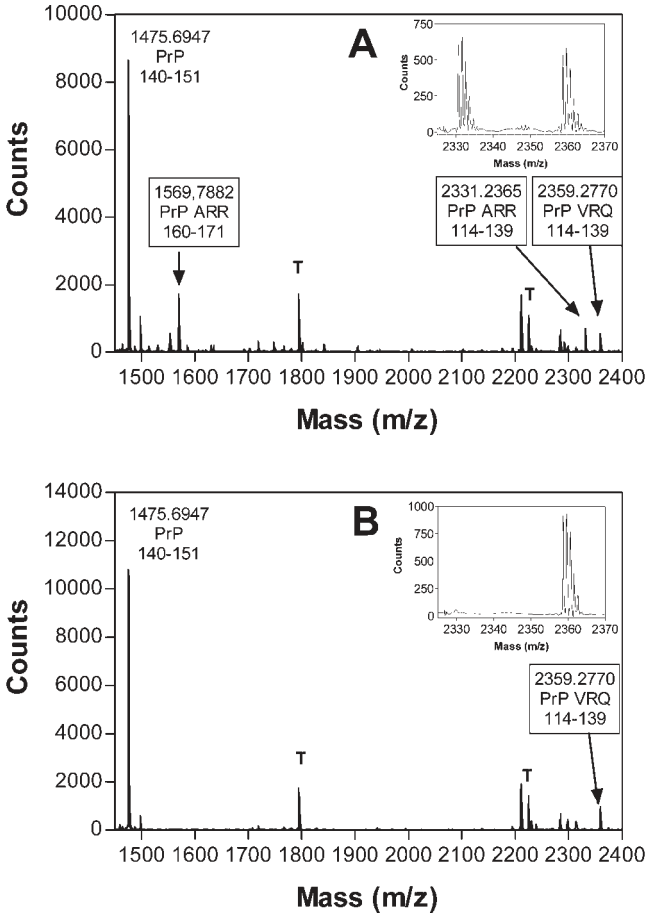

Figure 6. MALDI-TOF mass spectra in positive reflectron mode of the in-gel tryptic digests of immunoprecipitated PrP^{C} (A) and PrP^{Sc} (B) from an ARR/VRQ sheep brain homogenate. The same homogenate from a scrapie-infected brain was: (i) not treated with PK and not denatured (A), (ii) treated with PK and denatured (B) before immunoprecipitation with mAb Sha-31. Insets are the isotopic resolution of peptide 114–139 peaks, which contain the polymorphism site A136 or V136. Masses were calibrated with trypsin auto-digestion peptides 842.51 Da and 2211.1046 Da. Peaks labelled T result from trypsin auto-digestion.

When analysing a brain homogenate from a VRQ/ARR animal naturally infected with scrapie, we observed that only the V136 variant is found in the PK-resistant form of PRP (see Fig. 6, peptide 114–139). This result is confirmed by the presence of the 160–171 peptide in the PrP^{C} preparation and its absence from the PrP^{Sc} preparation (see Fig. 6). Thus the PrP^{Sc} fraction is entirely composed of the VRQ allele, showing that the ARR allele is not involved at all in the pathologic process leading to the accumulation of PrP^{Sc}.

DISCUSSION

Due to the importance of PrP polymorphisms in sheep which condition the susceptibility to TSEs and the pathogenesis of the disease (duration of the incubation period, distribution of PrPsc in the peripheral tissues), it was critical to develop methods to monitor the different allelic forms of the protein in tissues. This was particularly critical for heterozygous animals since, so far, no methods have been available to evaluate the respective contribution of each variant of the protein in the PrP^{C} or PrP^{Sc} form. In this paper we describe an integrated approach, which includes an efficient and rapid purification of PrP (both as PrP^{C} and PrP^{Sc}) and a MALDI-TOF analysis of the tryptic fragments generated from the purified protein.

Because of the low abundance of PrP in brain tissues (few μg/g of fresh tissue), the purification step is absolutely necessary and this work shows that it is possible to obtain an almost pure protein by combining efficient immunoprecipitation with SDS-PAGE. Measurements on different mixtures of VRQ and ARQ recombinant protein have shown that MALDI-TOF analysis of in-solution tryptic digests allows either a relative determination of the two variants or an absolute determination of each one by reference to an internal standard (C-terminally ^{18}O-labelled peptide) added to the tryptic digest.

Despite the low recovery (about 30%) of the tryptic peptides generated by in-gel digestion, a precise MALDI-TOF analysis can be performed on PrP variants (ARQ/VRQ

or ARR/VRQ) so as to determine the ratio of these allelic forms in brain tissues.

By analysing the brain from a heterozygous (ARQ/VRQ) scrapie-infected animal (Langlade strain), we showed that the VRQ variant accumulates preferentially in the PrP^{Sc} form (SAF) of the protein, while the two variants are expressed in equivalent amounts in the PrP^{C} form. This is perfectly in line with the increased sensitivity of homozygous VRQ/VRQ animals with regards to scrapie infection, compared with homozygous ARQ/ARQ animals, and indicates that the VRQ variant is more prone to convert from PrP^{C} into PrP^{Sc}.

A more contrasted result was observed by analysing a brain homogenate from a VRQ/ARR animal infected with scrapie since, in this situation, PrP^{Sc} was solely composed of the VRQ allele. Once again, this is perfectly in line with the resistance provided by the ARR allele.

A more detailed analysis of the differential behaviour of heterozygous ARQ/VRQ and VRQ/ARR animals compared with homozygotes (VRQ/VRQ and ARQ/ARQ) in the context of scrapie infection will be presented elsewhere (O. Andréoletti, in preparation).

In this work we have restricted our application to the study of the polymorphism in position 136 (A or V), but this approach can be extended to other polymorphic sites (154 R/H and 171 R/Q) and examples will be given elsewhere (O. Andréoletti, in preparation). Taken together, the two examples presented in this paper clearly illustrate the value of MALDI-TOF analysis combined with appropriate immunopurification techniques in seeking a precise understanding of the influence of PrP polymorphism on TSE pathogenesis.

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