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Prion protein allotype profiling by mass spectrometry*

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Abstract: Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative pathologies characterized by the formation in the central nervous system of the amyloid protein PrP^{Sc}, which derives from a cellular precursor called PrP^{c}. Epidemiological and laboratory studies have shown that in species where the PrP^{c} gene is polymorphic, the genotype composition is an important factor for the development of the disease. Identification of PrP^{Sc} allotypes accumulated in the brain during the disease proved valuable to investigate whether these polymorphisms are critical for the pathological conversion. These analyses are complicated by the heterogeneity and the insolubility of the prion amyloid extracted from affected brains, which have been obviated by extensive digestion of extracted fractions and analysis of peptide fragment composition. We have developed an optimized protocol of liquid chromatography/mass spectrometry (LC/MS) that can reliably map PrP peptides in digested fractions with a low PrP^{Sc}/contaminants ratio. This approach has been successfully applied to the analysis of amyloidogenesis in experimentally infected PrP-heterozygous laboratory animals.

INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders affecting humans (e.g., Creutzfeldt–Jakob disease) and animals (e.g., scrapie of sheep) and characterized by the accumulation, in the central nervous system, of the pathological protein PrP^{Sc}, which may eventually aggregate into amyloid plaques [1]. PrP^{Sc} derives from a host-encoded protein (PrP^{c}), following a conformational modification whose mechanism is still unclear [2]. Humans are affected by several forms of TSEs with different etiologies: sporadic Creutzfeldt–Jakob disease (sCJD) apparently arises as a spontaneous disease, genetic forms (e.g., familial CJD) are invariably associated with mutations of the PrP gene, infectious forms derive from a documented episode of accidental transmission [1–4]. In sporadic and accidental forms, genotype composition of the PrP gene at the polymorphic position M129V has been established as an important factor for the development of the disease [4,5]. It was suggested that mutations and polymorphism of PrP may either directly facilitate the pathological transformation of PrP, or its binding to exogenous or endogenous factors that participate in this transformation [6]. To gain insight into this dilemma, PrP^{Sc} allotype profiling was attempted in heterozygous

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patients with sporadic CJD and familial TSEs, in order to understand whether an allotype-selective conversion occurred. These studies assessed that, similar to other amyloidoses, PrP^{Sc} deposits are either entirely composed of the mutant precursor [7–11] or a mixture of wild-type and mutant PrP^{Sc} allotypes [11–14], suggesting that only some mutations may directly push PrP^{c}-PrP^{Sc} conversion. In order to enlarge these studies to include amyloidogenesis in experimental TSE models, we attempted to develop a fast and sensitive analytical procedure to identify PrP^{Sc} allotypes, that can be applied to small amounts of material extracted from affected brains. To this aim, we have recently approached an electrospray mass spectrometric identification of PrP^{Sc} isoforms through their reporter peptides. The optimized method has been applied to build PrP^{Sc} allotype profiling in PrP heterozygous mice experimentally infected either with the mouse-adapted scrapie strain 139A, or with mouse-passaged human TSE (hTSE) inocula.

MATERIALS AND METHODS

Infection of animals

Scrapie 139A inoculum was prepared as a 10 % suspension in phosphate-buffered saline (PBS) of brains from scrapie-affected C57BL mice. Mouse-passaged human TSEs inocula were prepared as 1 % brain homogenates from C3H mice infected with brain material from one familial CJD case bearing mutation V210I of PrP, and from two sporadic human CJD patients, either methionine homozygous or heterozygous at the polymorphic PrP residue 129 (the 129 homozygous sCJD and the familial CJD cases are described in ref. 14). Four groups of 60 hTSEs or 20 (strain 139A) weanling PrP heterozygous mice (produced by crossing mouse strains C57BL × I/LnJ) were infected by intracerebral injection with 30 µl of each inoculum. The animals were kept with food and water *ad libitum* and weekly observed until they showed clinical signs of disease. When neurological symptoms were clearly apparent, the animals were killed by CO_2 asphyxia, and their brains were collected and immediately frozen for the purification of PrP^{Sc}.

Frozen hamster 263K brains were already available in our laboratory.

Prion allotype characterization

PrP^{Sc} was extracted from frozen brains according to Diringer and colleagues [15]. The infectious fraction containing the protease-resistant core fragment of PrP^{Sc} (called PrP27-30) was inactivated with 1 ml 80 % formic acid for 3 h at 4 °C. After centrifugation at 14 000 × g for 30 min at 4 °C, the supernatant was collected, washed in water, and finally buffered at pH 7.8 with 0.5 M ammonium bicarbonate. PrP27-30 suspension was dried, resuspended in 50 µl of digestion buffer (1 mM EDTA, 25 mM TrisHCl pH 8.25, and 1 % octyl-β-glucopyranoside), and incubated with 2.5 µg of endoprotease LysC (Roche, Basel, Switzerland) at 37 °C for 24 h. Proteolysis was stopped, and amyloid peptides were disaggregated by dilution with 50 µl of 20 % trifluoroacetic acid.

Peptide mixture was then separated by reverse-phase high-performance liquid chromatography (RP-HPLC) on a LabFlow 4000 apparatus (LabService Analytica, Bologna, Italy) equipped with a C_{18} mass spec column (2.1 × 220 mm, 5 µm particle size, 300 Å pore size, Vydac, Hesperia, CA, USA). Elution was achieved using a linear gradient of 1–60 % acetonitrile containing 0.05 % trifluoroacetic acid in 60 min, at a constant flow rate of 150 µl/min. The column effluent was analyzed by an electrospray ion trap mass spectrometer (ES-IT, mod. LCQ, ThermoFinnigan, San Jose, CA, USA). Positive mass spectra of parent ions were collected on-line both over the mass range of 150–2000 atomic mass units (amu) in the full mass mode and by monitoring selected-ion masses (SIM mode). Endoprotease LysC PrP peptides TNXK, in which X is M in the hamster and L or F in the mouse prion, were used as reporters of the relative PrP^{Sc} allotypes. According to the expected mass spectrometric signals of the relative target monoisotopic ions, mass ranges assayed in SIM mode were 491–495 in the hamster PrP^{Sc}

analysis, and 473–477 and 507–511 in the 139A mouse PrP^{Sc} analysis. To improve analytical sensitivity, allotype profiling of hTSE-infected mice was established using a mass range of 472–515. In the hamster PrP analysis only, positive MS/MS spectra were also collected on-line after isolation and fragmentation of the reporter ion at *m/z* 493.2 over a mass range of 150–500 amu, and, according to the fragmentation pattern, by ion current monitoring in the range of its selected b_2 product ion at *m/z* 216.0 (selective reaction monitoring, SRM mode, *m/z* range 216.0 ± 3). In all MS analyses, spray voltage was 5.15 kV and the heated capillary was maintained at 260 °C. Total ion current (TIC), SIM, and SRM chromatographic traces were automatically built by the Xcalibur software provided with the instrument.

RESULTS

The TSE model represented by the 263 K scrapie strain in hamster (a species where the PrP gene has no allelic variants) is a convenient source of material to optimize mass spectrometric analysis protocols because of its high yield of PrP^{Sc} (about 100 µg/g fresh brain tissue [15]). In Fig. 1, the hamster PrP amino acid sequence is aligned with those of the two mouse PrP allotypes. Endoprotease LysC treatment of hamster PrP27-30 yields a mixture of proteolytic fragments that contains peptides 107TNMK110 and 186QHTVTTTTK194, bolded in Fig. 1. These peptides encompass the mouse polymorphic residues 109 (L or F) and 190 (T or V), which, in this paper, will be referred to as 108 and 189, according to the mouse primary structure as usually reported in the literature. These peptides could be selected as reporters for the detection of the relative PrP^{Sc} allotype.

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                10         20         30         40         50         60         70         80         90
                 |          |          |          |          |          |          |          |          |
Hamster MANLSYWLLA LFVAMWTDVG LCKKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGGGTWGQP HGGGWGQPHG GGWGQPHGGG WGQPHGGGWG
Mouse a MANLGYWLLA LFVTMWTDVG LCKKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGG-TWGQP HGGGWGQPHG GSWGQPHGGS WGQPHGGGWG
Mouse b MANLGYWLLA LFVTMWTDVG LCKKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGG-TWGQP HGGGWGQPHG GSWGQPHGGS WGQPHGGGWG

               100        110        120        130        140        150        160        170        180
                 |          |          |          |          |          |          |          |          |
Hamster QGGGTHNQWN KPSKPKTNMK HMAGAAAAGA VVGGLGGYML GSAMSRPMMH FGNDWEDRYY RENMNRYPNQ VYYRPVDQYN NQNNFVHDCV
Mouse a QGGGTHNQWN KPSKPKTNLK HVAGAAAAGA VVGGLGGYML GSAMSRPMIH FGNDWEDRYY RENMYRYPNQ VYYRPVDQYS NQNNFVHDCV
Mouse b QGGGTHNQWN KPSKPKTNFK HVAGAAAAGA VVGGLGGYML GSAMSRPMIH FGNDWEDRYY RENMYRYPNQ VYYRPVDQYS NQNNFVHDCV

               190        200        210        220        230        240        250
                 |          |          |          |          |          |          |
Hamster NITIKQHTVT TTTKGENFTE TDIKIMERVV EQMCTTQYQK ESQAYYDGRR SS-AVLFSSP PVILLISFLI FLMVG
Mouse a NITIKQHTVT TTTKGENFTE TDVKMMERVV EQMCVTQYQK ESQAYYDGRR SSSTVLFSSP PVILLISFLI FLIVG
Mouse b NITIKQHTVV TTTKGENFTE TDVKMMERVV EQMCVTQYQK ESQAYYDGRR SSSTVLFSSP PVILLISFLI FLIVG
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Fig. 1 Alignment of hamster and mouse PrP amino acid sequences. Discriminant residues for the two mouse allotypes are underlined. Endoprotease LysC peptides used as reporter ions are bolded. Gaps (–) in position 55 in the mouse and in position 233 in the hamster sequences, respectively, are introduced to maximize similarity.

As expected, the high complexity of the sample precluded any peptide identification when the HPLC eluate was monitored by total ion current in the full mass mode (Fig. 2A). However, the hamster prion peptide 107–110 could be detected using a SIM mode (Fig. 2B). The averaged mass spectrum acquired in the narrow scan range of 491–495 amu for the intact ion and its fragmentation pattern are both in perfect agreement with that calculated for the hamster prion reporter peptide (Figs. 2C and 2D). On the contrary, peptide 186–194 gave only a weak signal, probably due to its chemicophysical properties (data not shown). For this reason, in the following analyses we considered only peptide 107–110 as PrP reporter peptide ion.

Going one step further on this approach, we attempted to identify the PrP reporter peptide directly monitoring for the b_2 product ion (*m/z* of 216.0) according to the fragmentation pattern in Fig. 2D. As known, this latter mode of MS analysis is generally referred to as SRM mode. As indicated in Fig. 3, unequivocal detection of the hamster prion in the complex matrix was obtained.

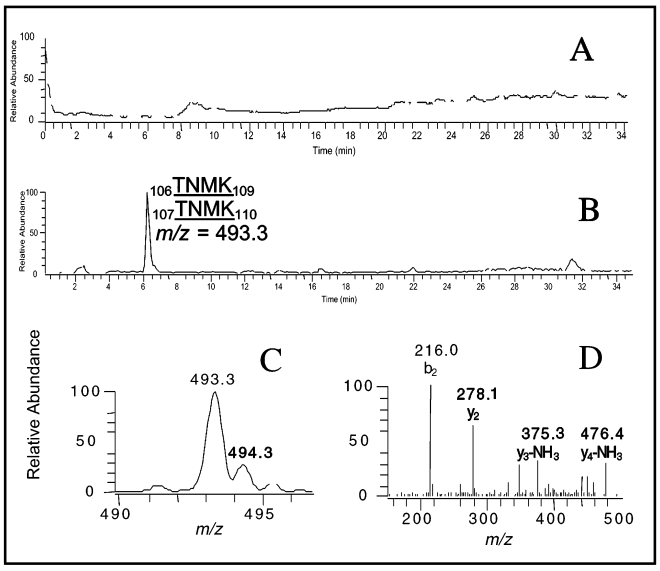

Fig. 2 Mass spectrometric detection of the hamster prion. HPLC separation of endoprotease LysC peptides monitoring by total ion current (panel A) and by selective ion monitoring for hamster PrP reporter peptide 107–110 (SIM mode, panel B). Peptide ion *m*/*z* and measured signal-to-noise values are also indicated with the peptide sequence attributed above the chromatographic peak. Panel C: averaged mass spectrum acquired over 491–495 amu for the chromatographic peak detected by the SIM mode. Panel D: averaged MS/MS spectrum of the parent ion in panel C. Fragment ion identifications are also reported.

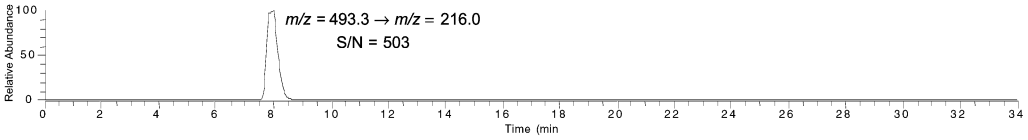

Fig. 3 Mass spectrometric detection of the hamster prion. HPLC separation of endoprotease LysC peptides monitoring by selected ion current collected in the *m*/*z* range 215.0–217.0 amu after selective isolation and fragmentation of the parent ion (SRM mode). Selected parent (before arrow) and product (after arrow) ions are reported, as well as the measured signal to noise value.

We applied the same approach to the characterization of PrP^{Sc} allotypes accumulating in the brain of PrP heterozygous mice experimentally infected with the scrapie strain 139A. The PrP27-30 enriched fraction was analyzed monitoring simultaneously for the two allotype-specific discriminant peptide ions (murine PrP sequence 106TNXK109). As shown in Fig. 4, the presence of both prion allotypes in 139A mouse brain was demonstrated by the strong signals generated by the 108L and 108F reporter peptides. Chromatographic peaks were also on-line analyzed in term of averaged mass spectrum and fragmentation pattern, which confirmed the peptide attribution (data not shown).

We have applied our approach also to the characterization of PrP^{Sc} allotypes produced after secondary experimental transmission of three human TSE cases to heterozygous mice. Allotype profiles were established with the SIM mode liquid chromatography/mass spectrometry (LC/MS) approach, monitoring in a narrow mass range that includes both mass values expected for the two murine reporter peptides encompassing residue 108. Even with a low signal-to-noise ratio, mainly due to the presence of small amounts of PrP^{Sc} in the preparation, again both allotypes of mouse PrP were found to accumulate (Fig. 5) in all of the samples tested.

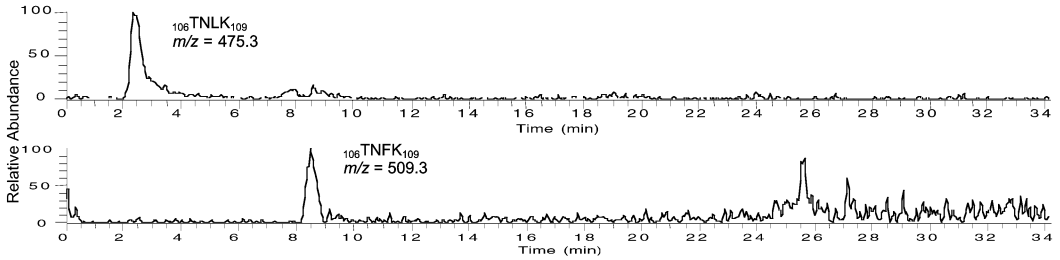

Fig. 4 Mass spectrometric detection of the two prion allotypes from the brain of heterozygous mice infected with scrapie. SIM mode mass spectrometric analysis of HPLC elution of endoprotease LysC peptides, on-line monitoring by ion current in the *m*/*z* range 473–477 amu (panel A) and 507–511 amu (panel B). The mass values measured and the PrP peptide sequences attributed are also indicated.

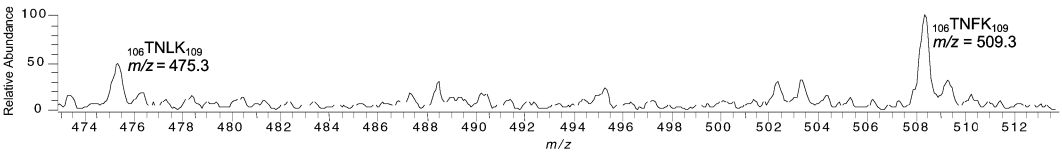

Fig. 5 Mass spectrometric detection of the two prion allotypes from the brain of heterozygous mice, after a hTSE experimental transmission. Averaged mass spectrum obtained over 472–515 amu from the endoprotease LysC peptide mixture from one of the prion positive samples in the elution time range 2–18 min. The mass value measured and the peptide sequence attributed are also indicated.

DISCUSSION

Characterization of PrP^{Sc} allotypes deposited in the brain of a TSE-affected subject represents a hard analytical problem. PrP^{Sc} is an insoluble protein with a high propensity to aggregate and virtually impossible to obtain in a pure form [16]. Extraction procedures usually yield a poorly soluble fraction containing a complex mixture of PrP27-30 isoforms with different levels of glycosylation [17]. Contaminant molecules comprise a poorly characterized proteinaceous material, traces of nucleic acid, and glycosaminoglycans [15,18,19]. Moreover, the isolation procedures generally introduce significant amount of salts and detergents that, owing to the poor solubility of PrP27-30 in organic solvents, cannot be easily removed by desalting chromatographic procedures. Most PrP polymorphisms and mutations are located in the central or in the C-terminal portion of the sequence [1], therefore, N-terminal Edman sequencing of the intact molecule can be only sporadically used for allotype identification [7]. For these reasons, prion protein isoforms have been generally identified after proteolytic treatments, which reach the double goal of solubilizing and producing discriminant peptides of limited size that can be easily studied by different analytical techniques. Moreover, the choice of the analytical procedure must consider the low amount of PrP27-30 that can be recovered after purification (up to 50 μg/g of brain in hamster, but as low as 2–5 μg/g in heterozygous mice infected with hTSEs). The LC-MS approach here proposed allowed us to detect the prion protein accumulated in heterozygous mice infected with different TSE strains with a good level of sensitivity and specificity. The interface between a chromatographic separation and the electrospray mass spectrometer equipped with the ion trap detector provided the necessary set-up to sustain a fast and sensitive identification of selected prion peptides in their complex matrix. The MS analysis in SIM mode allowed their detection with high signal-to-noise values, whereas the accuracy of this approach was ensured by the on-line structural characterization of the chromatographic peaks both by their mass spectrum acquired in the SIM narrow range and their fragmentation pattern. When enough PrP27-30 was available, all these MS analyses were performed on the same chromatographic peak. Identification of the target peptide was also obtained by its entrapment,

fragmentation, and subsequent monitoring for a selected product ion. As expected, results obtained in the SRM mode on hamster PrP27-30 clearly indicated an increase in selectivity, but a reduced sensitivity. The SIM mode of analysis was therefore selected to characterize PrP27-30-enriched fractions from mouse TSE brains. This experimental setting clearly discriminated the presence of both PrP^{Sc} allotypes in the brain of heterozygous mice infected with the scrapie strain 139A.

Western blot analysis of the PrP^{Sc} material from mice infected with human TSEs showed a lower concentration of the prion protein in comparison with the 139A strain. To increase further the PrP^{Sc} profiling sensitivity, peptide elution was only monitored in a selected mass range, encompassing the mass values expected for the two allotype peptides containing the mouse codon 108. With this protocol, masses corresponding to both reporter peptides could be unambiguously detected at the expected elution time range, suggesting that even in these three mouse-passaged human TSEs, two PrP^{Sc} allotypes are produced during the disease. The presence of both PrP allotypes in amyloid fractions extracted from heterozygous mice infected with hTSE inocula suggests that molecular pathogenesis produced by these TSE forms in this host does not differ significantly. This is in agreement with the clinical and pathological similarities observed after the primary passage of these inocula in C3H mice [M. Pocchiari, unpublished data].

From the methodological point, these results, as a whole, suggest that the proposed analytical approach can be successfully used for the identification of PrP^{Sc} allotypes when as low as 2–5 μg of material are available. However, the elective strategy to improve the comprehension of TSE amyloidogenesis requires an accurate quantification of the two allotypes. For this reason, we intend to implement the LC/MS approach by selecting suitable standards.

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REFERENCES

1. S. Prusiner. *Proc. Natl. Acad. Sci. USA* **95**, 13363–13383 (1998).
2. F. Cardone and M. Pocchiari. In *Prions and Brain Diseases in Animals and Humans*, D. R. O. Morrison (Ed.), pp. 245–252, Plenum Press, New York (1998).
3. M. Pocchiari. *Mol. Aspects Med.* **15**, 195–291 (1994).
4. P. Brown, M. Preece, J. P. Brandel, T. Sato, L. McShane, I. Zerr, A. Fletcher, R. G. Will, M. Pocchiari, N. R. Cashman, J. H. d'Aignaux, L. Cervenakova, J. Fradkin, L. B. Schonberger, S. J. Collins. *Neurology* **55**, 1075–1081 (2000).
5. A. Alperovitch, I. Zerr, M. Pocchiari, E. Mitrova, J. de Pedro Cuesta, I. Hegyi, S. Collins, H. Kretzschmar, C. van Duijn, R. G. Will. *Lancet* **353**, 1673–1674 (1999).
6. B. Chesebro. *Science* **279**, 42–43 (1998).
7. P. Barbanti, G. Fabbrini, M. Salvatore, R. Petraroli, F. Cardone, B. Maras, M. Equestre, G. Macchi, G. L. Lenzi, M. Pocchiari. *Neurology* **47**, 734–741 (1996).
8. T. Kitamoto, K. Yamaguchi, K. Doh-ura, J. Tateishi. *Neurology* **41**, 306–10 (1991).
9. F. Tagliavini, F. Prelli, M. Porro, G. Rossi, G. Giaccone, M. R. Farlow, S. R. Dlouhy, B. Ghetti, O. Bugiani, B. Frangione. *Cell* **79**, 695–703 (1994).
10. F. Tagliavini, P. M. Lievens, C. Tranchant, J. M. Warter, M. Mohr, G. Giaccone, F. Perini, G. Rossi, M. Salmona, P. Piccardo, B. Ghetti, R. C. Beavis, O. Bugiani, B. Frangione, F. Prelli. *J. Biol. Chem.* **276**, 6009–6015 (2001).
11. S. G. Chen, P. Parchi, P. Brown, S. Capellari, W. Zou, E. J. Cochran, C. L. Vnencak-Jones, J. Julien, C. Vital, J. Mikol, E. Lugaresi, L. Autilio-Gambetti, P. Gambetti. *Nat. Med.* **3**, 1009–1015 (1997).

12. B. Ghetti, P. Piccardo, M. G. Spillantini, Y. Ichimiya, M. Porro, F. Perini, T. Kitamoto, J. Tateishi, C. Seiler, B. Frangione, O. Bugiani, G. Giaccone, F. Prelli, M. Goedert, S. R. Dlouhy, F. Tagliavini. *Proc. Natl. Acad. Sci. USA* **93**, 744–748 (1996).
13. R. Gabizon, G. Telling, Z. Meiner, M. Halimi, I. Kahana, S. B. Prusiner. *Nat. Med.* **2**, 59–64 (1996).
14. M. C. Silvestrini, F. Cardone, B. Maras, P. Pucci, D. Barra, M. Brunori, M. Pocchiari. *Nat. Med*. **3**, 521–525 (1997).
15. H. Diringer, M. Beekes, M. Özel, D. Simon, I. Queck, F. Cardone, M. Pocchiari, J. W. Ironside. *Intervirology* **40**, 238–246 (1998).
16. R. K. Meyer, M. P. McKinley, K. A. Bowman, M. B. Braunfeld, R. A. Barry, S. B. Prusiner. *Proc. Natl. Acad. Sci. USA* **83**, 2310–2314 (1986).
17. G. Zanusso, P. G. Righetti, S. Ferrari, L. Terrin, A. Farinazzo, F. Cardone, M. Pocchiari, S. Monaco. *Electrophoresis* **23**, 347–355 (2002).
18. A. D. Snow, T. N. Wight, D. Nochlin, Y. Koike, K. Kimata, S. J. DeArmond, S. B. Prusiner. *Lab. Invest.* **63**, 601–11 (1990).
19. N. Stahl, M. A. Baldwin, D. B. Teplow, L. Hood, B. W. Gibson, A. L. Burlingame, S. B. Prusiner. *Biochemistry* **32**, 1991–2002 (1993).