Fibril Formation of the Rabbit/Human/Bovine Prion Proteins

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Running Title: Fibrillization of the Rabbit/Human/Bovine PrP

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Supplemental Data

EXPERIMENTAL PROCEDURES

Materials

The crowding agents, Ficoll 70 and dextran 70, were purchased from Sigma-Aldrich (St. Louis, MO). Thioflavin T (ThT) was also obtained from Sigma-Aldrich. Guanidine hydrochloride was obtained from Promega (Madison, WI). Proteinase K, Triton X-100, and urea were purchased from Ameresco (Solon, OH). All other chemicals used were made in China and were of analytical grade.

Expression and Purification of Prion Proteins

The rabbit/human/bovine PrP cDNA was subcloned into pET30a vector. Triple mutant G99N/L137I/S173N of the rabbit PrP was generated based on the rabbit PrP plasmid using the QuickChange PCR method. Recombinant full-length rabbit/human/bovine prion proteins were expressed in *E. coli*, isolated on a Ni-NTA agarose column, and further purified by HPLC on a C4 reversed-phase column (Shimadzu, Kyoto, Japan) as described by Bocharova and co-workers (17). Purified rabbit/human/bovine prion proteins were confirmed by SDS-PAGE and mass spectrometry to be single species with an intact disulfide bond. The concentrations of the rabbit PrP, human PrP, and bovine PrP were determined by their absorbance at 280 nm using the molar extinction coefficient values of 57,995, 57,995, and 63,495 M^{-1} cm^{-1}, respectively, deduced from the composition of the proteins online.

Fibril Formation and Thioflavin T Binding Assays

A 2.5 mM ThT stock solution was freshly prepared in phosphate-buffered saline solution (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.0), and passed through a 0.22-μm pore size filter before use to remove insoluble particles. Under standard conditions, a stock solution of the rabbit/human/bovine PrP in 6 M guanidine hydrochloride was diluted to a final concentration of 10 μM and incubated at 37°C in PBS buffer (pH 7.0) containing 1 M guanidine hydrochloride and 3 M urea in the absence and presence of different concentrations of crowding agents with continuous shaking at 220 rpm, and samples (50 μl) were diluted into PBS buffer containing 12.5 μM ThT, giving a final volume of 2.5 ml. The fluorescence of ThT was excited at 450 nm with a slit-width of 7.5 nm and the emission was measured at 480 nm with a slit-width of 7.5 nm on an LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT). The fluorescence intensity at 480 nm was averaged over 60 s to increase the signal-to-noise ratio of the measurements. Control experiments were performed to ensure that the crowding agents had no influence on the ThT binding assays for the rabbit/human/bovine PrP. Kinetic parameters were determined by fitting ThT fluorescence intensity *versus* time to a sigmoidal equation (20,28,36):

$$F = F_0 + (A + ct)/ \{1 + \exp[k(t_m - t)]\} \qquad (1)$$

where F is the fluorescence intensity, k is the rate constant for the growth of fibrils, and t_m is the time to 50% of maximal fluorescence. The initial baseline during the lag

time is described by F_0. The final baseline after the growth phase has ended is described by $A + ct$. The lag time is calculated as $t_m - 2/k$.

Seeding Experiments

Mature fibrils formed by the rabbit/human/bovine PrP were sonicated on ice using a probe sonicator for 30 s (interval of 5 s, 200 W) in order to produce the seeds used in the seeding experiments. The kinetics of fibril formation of samples with 0.5% (v/v) pre-formed seed fibrils at the initial time were monitored by ThT binding assays.

Sarkosyl-soluble SDS-PAGE

Amyloid formation of 20 μM rabbit/human PrP was carried out as state above, during the incubation time, 20 μl samples were taken out and added with 2.5 μl of 100 mM Tris-HCl (pH 7.0) and 2.5 μl of 20% Sarkosyl. The mixture were left at room temperature for 30 min, then mixed with 2X loading buffer (without β-mercaptoethanol and no heating) and separated by 15% SDS-PAGE. Gels were stained by Coomassie Blue.

Transmission Electron Microscopy

The formation of fibrils by the rabbit/human/bovine PrP was confirmed by electron microscopy of negatively stained samples. The incubation time was chosen within a time range of the plateau of each kinetic curve of ThT fluorescence shown in Fig. 1. Sample aliquots of 10 μl were placed on copper grids and left at room temperature for 1-2 min, rinsed twice with H_2O, and then stained with 2% (w/v) uranyl acetate for another 1-2 min. The stained samples were examined using an H-8100 transmission electron microscope (Hitachi, Tokyo, Japan) operating at 100 kV or an FEI Tecnai G2 20 transmission electron microscope (Hillsboro, OR) operating at 200 kV.

Atomic Force Microscopy

AFM was used to probe the structural order in the rabbit PrP aggregates with the aim to see if these are still fibrils or disordered complexes. Sample aliquots of 10 μl were deposited onto freshly cleaved mica, left on the surface for 15 min and rinsed with H_2O twice. Then the solution was blown off with compressed N_2 and dried air. AFM images were acquired in tapping mode with a SPM-9500 J3 scanning probe microscope (Shimadzu, Kyoto, Japan). Several regions of the mica surface were examined to confirm that similar structures existed through the sample.

Fourier Transform Infrared Spectroscopy

Attenuated total reflection FTIR spectra were recorded using a Nicolet 5700 FTIR spectrophotometer (Thermo Electron, Madison, WI). Rabbit/human/bovine PrP fibril samples were prepared in D_2O and FTIR spectra were recorded in the range from 400 to 4000 cm^{-1} at 4 cm^{-1} resolution. The sample was scanned 128 times in each FTIR measurement, and the spectrum acquired is the average of all these scans. Spectra were corrected for the D_2O and water vapors. To deconvolute the original absorbance spectra to individual peaks, the peak resolve procedure (OMNIC 8 software) was employed by setting the peak maxima at wavelengths taken from FTIR second derivative spectra. Measurements were made at 25^oC.

CD Measurements

Circular dichroism spectra were obtained by using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan) with a thermostated cell holder. Quartz cell with a 1 mm

light-path was used for measurements in the far-UV region. Spectra were recorded from 195 to 250 nm for far-UV CD. Rabbit/human/bovine PrP fibril samples were subjected to extensive dialysis against PBS buffer (pH 7.0) to remove guanidine hydrochloride and urea. The final concentration of the rabbit/human/bovine PrP was kept at 10 μM. The averaged spectra of several scans were corrected relative to the buffer blank or the buffer containing crowding agents. Measurements were made at 25^{o}C.

PK Digestion Assays

Rabbit/human/bovine PrP fibril samples were prepared in 100 mM Tris-HCl buffer (pH 7.5) and incubated with PK at a PK : PrP molar ratio of 1:1000 to 1:50 for 1 h at 37^{o}C. Digestion was stopped by the addition of 2 mM phenylmethylsulfonyl fluoride (PMSF), and samples were analyzed in 15% SDS-PAGE and detected by silver staining. Rabbit/human/bovine PrP fibril samples were also incubated with 4 μg/ml PK at a PK : PrP molar ratio of 1:100. Samples were taken and the reaction was quenched by the addition of 2 mM PMSF at digestion time of 10, 20, 30, 40, 50, and 60 min, respectively, and analyzed by 15% SDS-PAGE.

MALDI-TOF Mass Spectrometry

Rabbit/human/bovine PrP fibrils were digested by proteinase K for 40 min and dissolved in 100 mM Tris-HCl buffer (pH 7.5) containing 3 M guanidine hydrochloride and 0.5 M β-mercaptoethanol. The resultant products were concentrated and desalted using C4 ZipTips (Millipore, Bedford, MA) according to the manufacturer's protocol. Saturated sinapinic acid was used as the matrix and prepared in aqueous solution containing 45% acetonitrile and 0.1% trifluoroacetic acid. The sample was mixed with matrix on a ground steel target plate and allowed to air-dry at room temperature and then subjected to MALDI-TOF analysis. MALDI-TOF mass spectra were acquired on an UltraFlex I MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). An accelerating voltage of 25 kV was used. All spectra were recorded in the positive ion linear mode in the 5000-25000 *m/z* mass range. Ubiquitin, cytochrome *c*, and myoglobin were used as calibrants for data calibration.

Nano-LC-MS Analysis

Rabbit/human/bovine PrP fibrils were digested by proteinase K for 40 min and dissolved in 100 mM Tris-HCl buffer (pH 7.5) containing 3 M guanidine hydrochloride and 0.5 M β-mercaptoethanol. The resultant products were centrifuged at 17,000 × *g* for 5 min prior to analysis. The supernatants were analyzed by nano-LC on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA). 60 μg of peptide mixtures was pressure-loaded onto a 12-cm silica capillary column with filter packed with 10-cm PS/DVB polymer resin (Welch 5 μm Hydro-RP 800 Å, Shanghai, China). Such a polymer resin silica capillary column (100-μm id) was used as analysis column. The buffer solutions used were 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B). The column was first desalted with buffer A and then eluted by a nonlinear increasing gradient of buffers A to B during 50 min at 800 nl/min flow rate. The gradient contained: 5 min of 5% buffer B, 35 min of 25% buffer B, 5 min of 90% buffer B, and finally 5 min of 5% buffer B. The

LTQ-Orbitrap was operated in FTMS profile full MS mode with the Xcalibur software. Nano-ESI was accomplished with a spray voltage of 2.1 kV and a heated capillary temperature of 180^{o}C. Injection time was set to 500 ms and 2 micro scans were set in the Orbitrap in the 500-1700 *m/z* range with the resolution set to a value of 100,000.

Supplemental Data

TABLE S1

PK-digestion products of rabbit/human/bovine PrP fibrils identified by Nano-LC LTQ Orbitrap MS, with molecular masses higher than 10 kDa. The measured masses were compared with average theoretical masses using FindPept programs available at the Swiss-Prot Data Bank (http://www.expasy.ch/tools). The differences between measured and theoretical masses of the identified peptides were less than 3 Da. The molecular masses of PK digestion products were calculated as the mean of 3-7 peak maximum values ± S.E.

Rabbit PrP fibrils	Measured mass [a]	Theoretical mass [b]	Position [c]	Sequence
Fragment 1	11488.29 ± 0.43	11489.68	134-228	SRPLIHFGND·····AYQRA
Fragment 2	11034.46 ± 0.50	11036.14	138-228	IHFGNDYEDR·····AYQRA
Human PrP fibrils				
Fragment 1	15554.06 ± 2.08	15554.40	97-231	SQWNKPSKPK·····QRGSS
Fragment 2	15337.85 ± 0.56	15339.19	99-231	WNKPSKPKTN·····QRGSS
Fragment 3	15961.41 ± 2.99	15963.80	92-231	GGGTHSQWNK·····QRGSS
Fragment 4	13214.74 ± 2.05	13217.68	119-231	GAVVGGLGGY·····QRGSS
Fragment 5	13259.08 ± 1.40	13256.76	116-229	AAAGAVVGGL·····YYQRG
Fragment 6	13358.13 ± 0.85	13359.84	117-231	AAGAVVGGLG·····QRGSS
Fragment 7	13399.89 ± 0.94	13398.92	113-228	AGAAAAGAVV·····AYYQR
Fragment 8	13428.48 ± 2.31	13430.92	116-231	AAAGAVVGGL·····QRGSS
Fragment 9	12081.13 ± 1.27	12081.39	130-229	LGSAMSRPII·····YYQRG
Bovine PrP fibrils				
Fragment 1	15355.91 ± 0.29	15356.19	108-241	GQWNKPSKPK·····YQRGA
Fragment 2	15821.91 ± 0.33	15822.64	102-240	GQGGTHGQWN·····YYQRG
Fragment 3	13309.27 ± 0.81	13310.79	128-242	AAGAVVGGLG·····QRGAS
Fragment 4	13166.52 ± 1.51	13168.64	130-242	GAVVGGLGGY·····QRGAS
Fragment 5	11745.51 ± 1.03	11746.94	146-242	SRPLIHFGSD·····QRGAS
Fragment 6	11876.60 ± 1.23	11878.14	145-242	MSRPLIHFGS·····QRGAS
Fragment 7	11946.82 ± 0.94	11949.22	144-242	AMSRPLIHFG·····QRGAS

[a] Calculated mass for the observed molecular ion peptide $[M + nH]^{n+}$.

[b] Theoretical mass for the average of the considered molecular ion peptide $[M+ H]^{+}$.

[c] The location of the peptide in rabbit/human/bovine PrP sequence.

Supplemental Data

FIGURE S1. **Time-dependent SDS-PAGE analysis of sarkosyl-soluble human PrP incubated in 0 g/l (*A*) and 200 g/l (*B*) Ficoll 70.** Samples were taken and incubated with Tris-HCl buffer containing 2% sarkosyl for 30 min. Then the samples were mixed with 2 X loading buffer and separated by 15% SDS-PAGE. Gel was stained by Coomassie Blue R250.

FIGURE S2. **Time-dependent SDS-PAGE analysis of sarkosyl-soluble rabbit PrP incubated in 0 g/l (*A*) and 150 g/l (*B*) Ficoll 70.** Samples were taken and incubated with Tris-HCl buffer containing 2% sarkosyl for 30 min. Then the samples were mixed with 2 X loading buffer and separated by 15% SDS-PAGE. Gel was stained by Coomassie Blue R250.

FIGURE S3. **AFM images (1 μm × 1 μm) of rabbit PrP aggregates formed at physiological pH after incubation under different conditions.** Rabbit PrP samples were incubated for 8 h in the absence of a crowding agent (*A*) and in the presence of 150 g/l Ficoll 70 (*B*), respectively. The scale bars represent 500 nm.

FIGURE S4. **Comparison of the secondary structures of rabbit (*A*), human (*B*), and bovine (*C*) PrP fibrils by FTIR.** The FTIR spectra (*blue lines*) and the results of curve fitting (*red lines*) of the FTIR amide I' region of amyloid fibrils produced from 20 μM rabbit, human, and bovine prion proteins incubated for 12, 12, 16 h, respectively, in the absence of a crowding agent. Peaks corresponding to individual components of secondary structures are shown by *black lines*.

FIGURE S5. **Time-dependent proteinase K-digestion assays of rabbit (*A*), human (*B*), and bovine (*C*) PrP fibrils.** Samples were treated with 4 μg/ml PK at 37°C at a PK : PrP molar ratio of 1:100. Samples were taken and the reaction was quenched by the addition of 2 mM PMSF at digestion time of 10 min (*lane 2*), 20 min (*lane 3*), 30 min (*lane 4*), 40 min (*lane 5*), 50 min (*lane 6*), and 60 min (*lane 7*), respectively. The controls with zero protease in the absence of a crowding agent were loaded in *lane 1*. Protein molecular weight markers were loaded on *lane M*: restriction endonuclease Bsp98 I (25.0 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). Amyloid fibrils were produced from the rabbit, human, and bovine prion proteins incubated for 12, 12, 16 h, respectively, in the absence of a crowding agent. Protein fragments were separated by SDS-PAGE and detected by silver staining.

FIGURE S6. **Nano-LC-MS analysis of proteinase K-resistant fragments of rabbit PrP fibrils.** Amyloid fibrils, produced from the rabbit PrP incubated for 12 h, were treated with 4 μg/ml PK for 40 min at 37^{o}C and analyzed by nano-LC on a high-resolution LTQ Orbitrap XL mass spectrometer. The total ion chromatogram of PK-digestion products of rabbit PrP fibrils (*A*) eluted from a polymer resin silica

capillary column. Two PK-resistant fragments encompassing residues 134-228 (*B*, Fragment 1, measured mass 11488.29 Da, theoretical mass 11489.68 Da) and 138-228 (*B*, Fragment 2, measured mass 11034.46 Da, theoretical mass 11036.14) for rabbit PrP fibrils were identified by comparison of measured and theoretical masses.

FIGURE S7. **Nano-LC LTQ Orbitrap MS analysis of proteinase K-resistant core fragments of human PrP fibrils.** Amyloid fibrils, produced from human PrP incubated for 12 h in the absence of a crowding agent, were treated with 4 μg/ml PK for 40 min at 37^{o}C and analyzed by nano-LC on a high-resolution LTQ Orbitrap XL mass spectrometer with ESI ion source. Three PK-resistant core fragments encompassing residues 97-231 (*A*, Fragment 1, measured mass 15554.06 Da, theoretical mass 15554.40 Da), residues 92-231 (*A*, Fragment 2, measured mass 15337.85 Da, theoretical mass 15339.19 Da), and residues 99-231 (*B*, Fragment 3, measured mass 15961.41 Da, theoretical mass 15963.80 Da) for human PrP fibrils were identified by comparison of measured and theoretical masses. PK-digestion products of rabbit/human/bovine PrP fibrils identified by LTQ Orbitrap MS, with molecular masses higher than 10 kDa, are summarized in supplemental Table S1.

FIGURE S8. **Secondary structural changes of rabbit PrP isoforms in the presence of 150 g/l Ficoll 70 (*A*) or 150 g/l dextran 70 (*B*) monitored by far-UV CD.** *Curve a*: native rabbit PrP. *Curve b*: amyloid fibrils produced from the rabbit PrP incubated for 12 h.

FIGURE S9. **Effects of macromolecular crowding on amyloid formation of triple mutant G99N/L137I/S173N of the rabbit PrP.** Triple mutant of the rabbit PrP in the absence and presence of Ficoll 70, monitored by ThT fluorescence. All experiments were repeated at least once. The final concentration of the triple mutant was 10 μM. The crowding agent concentrations were 0 (*open square*), 150 g/l (*solid circle*), and 200 g/l (*solid triangle*), respectively. The data were fitted to a sigmoidal equation, and the *solid lines* represent the best fit.

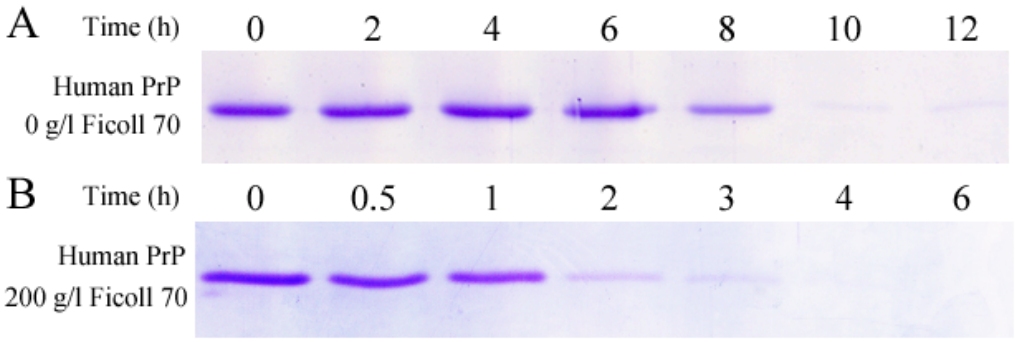
A
Time (h)
0
2
4
6
8
10
12
Human PrP
0 g/l Ficoll 70
B
Time (h)
0
0.5
1
2
3
4
6
Human PrP
200 g/l Ficoll 70

Figure S1

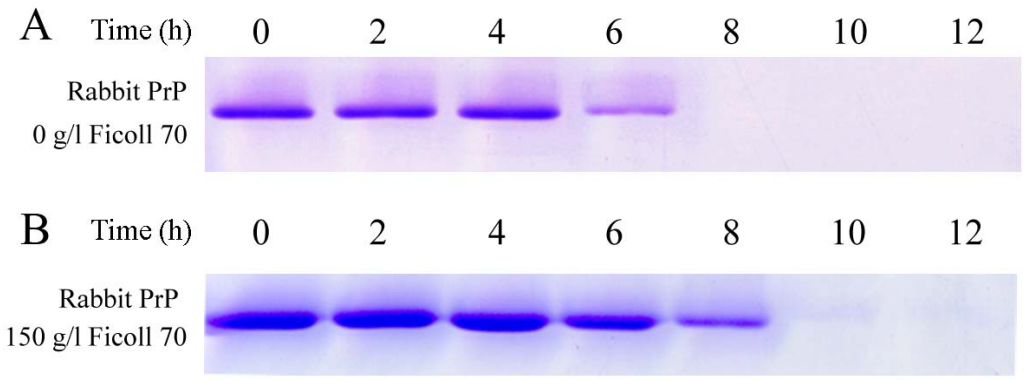
A
Time (h)
0
2
4
6
8
10
12
Rabbit PrP
0 g/l Ficoll 70
B
Time (h)
0
2
4
6
8
10
12
Rabbit PrP
150 g/l Ficoll 70

Figure S2

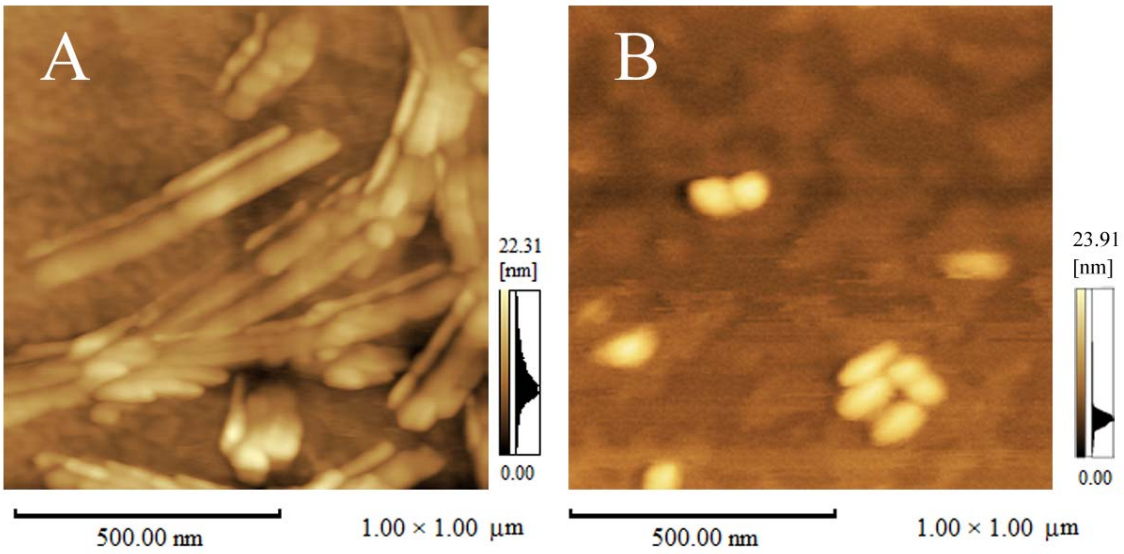
A
22.31
[nm]
0.00
500.00 nm
1.00 × 1.00 µm
B
23.91
[nm]
0.00
500.00 nm
1.00 × 1.00 µm

Figure S3

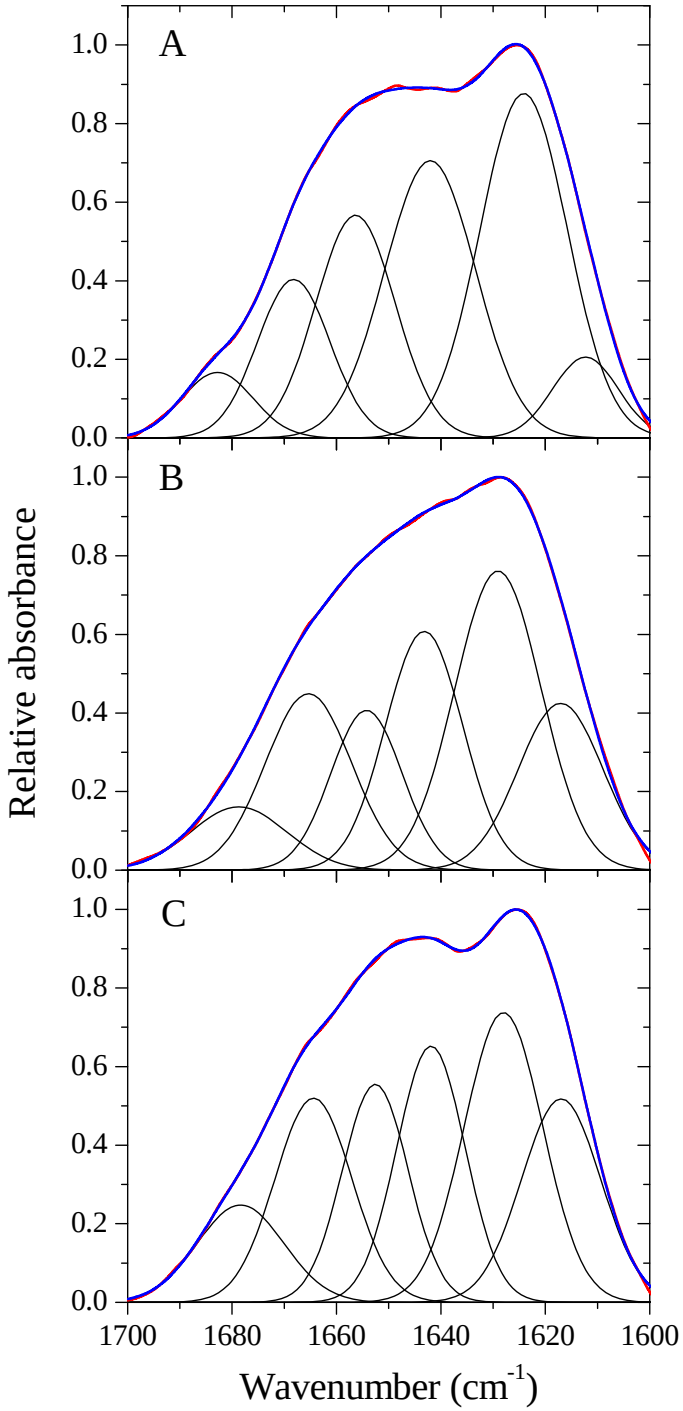
A
B
C
1.0
0.8
0.6
0.4
0.2
0.0
1700
1680
1660
1640
1620
1600
Relative absorbance
Wavenumber (cm-1)

Figure S4

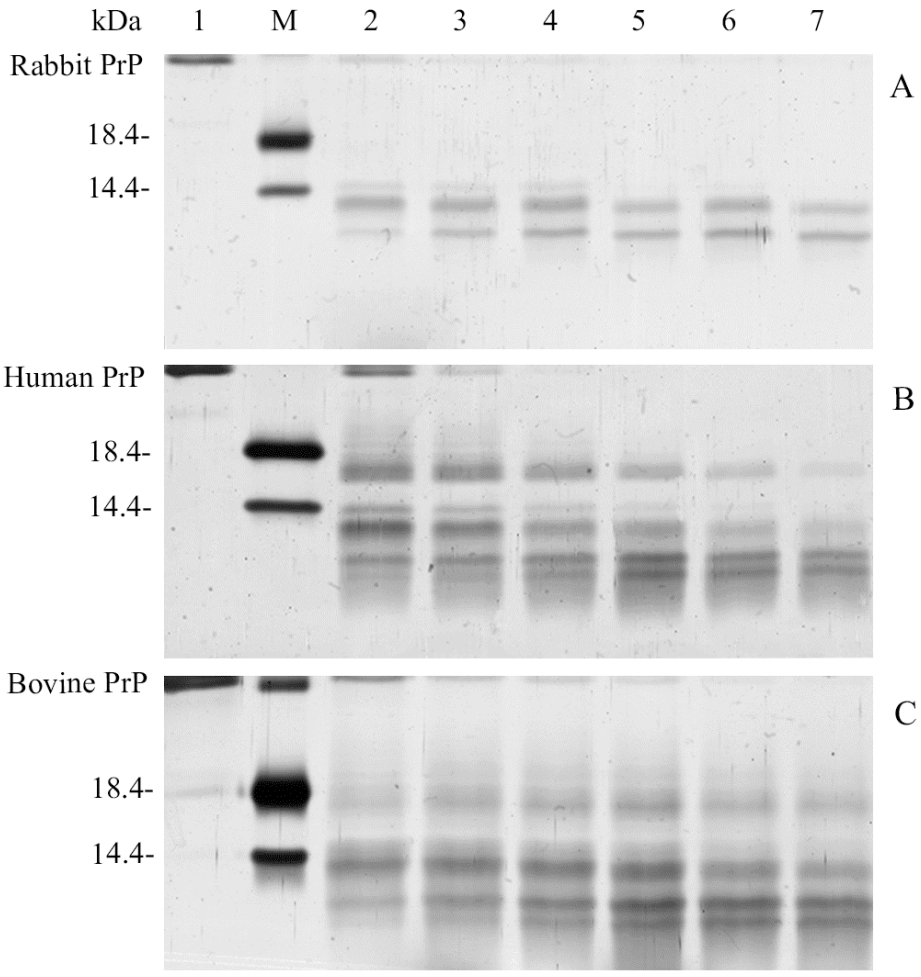
kDa
1
M
2
3
4
5
6
7
Rabbit PrP
18.4-
14.4-
A
Human PrP
18.4-
14.4-
B
Bovine PrP
18.4-
14.4-
C

Figure S5

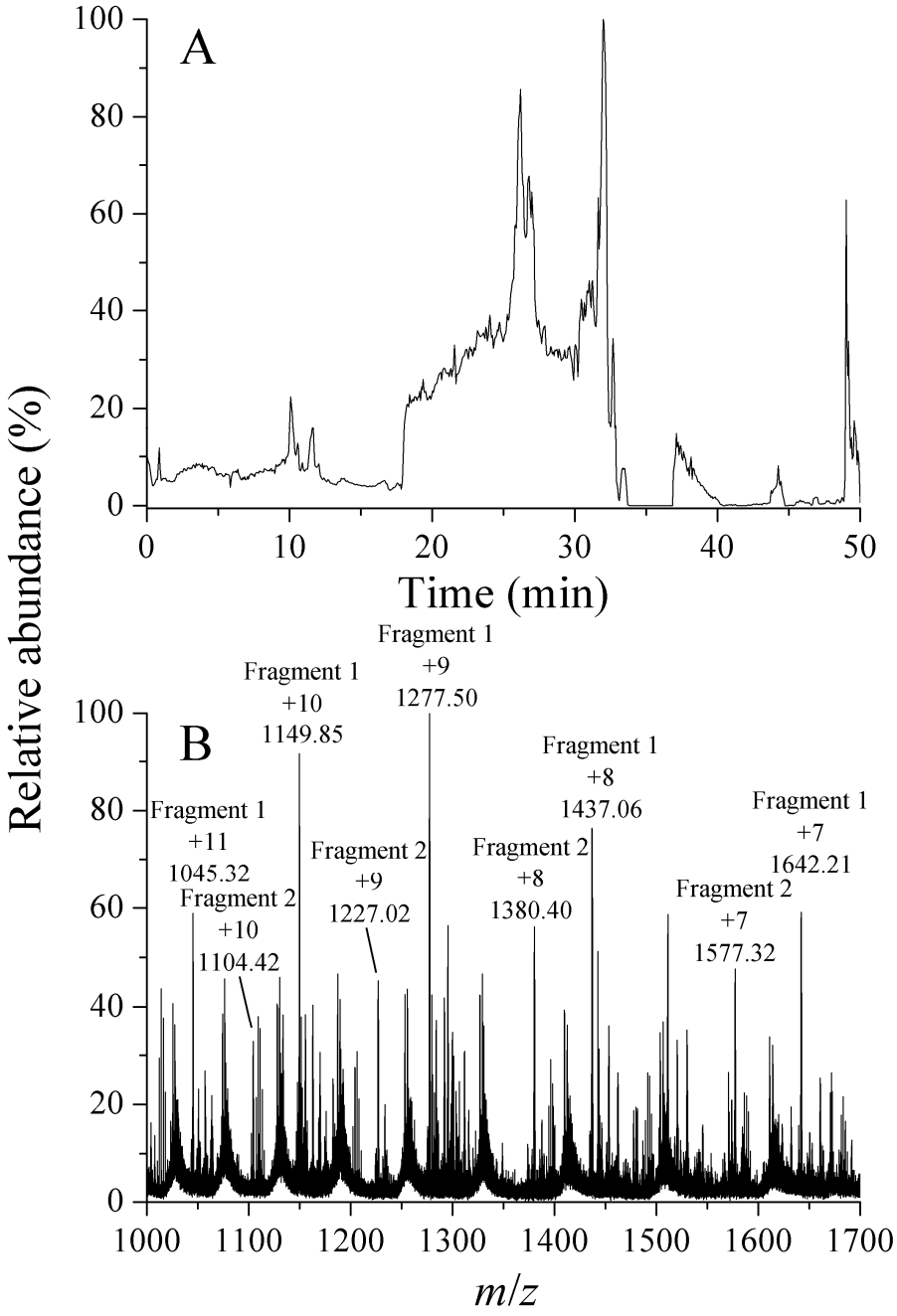
A
Relative abundance (%)
Time (min)
B
Fragment 1 +11 1045.32
Fragment 2 +10 1104.42
Fragment 1 +10 1149.85
Fragment 2 +9 1227.02
Fragment 1 +9 1277.50
Fragment 2 +8 1380.40
Fragment 1 +8 1437.06
Fragment 2 +7 1577.32
Fragment 1 +7 1642.21
m/z

Figure S6

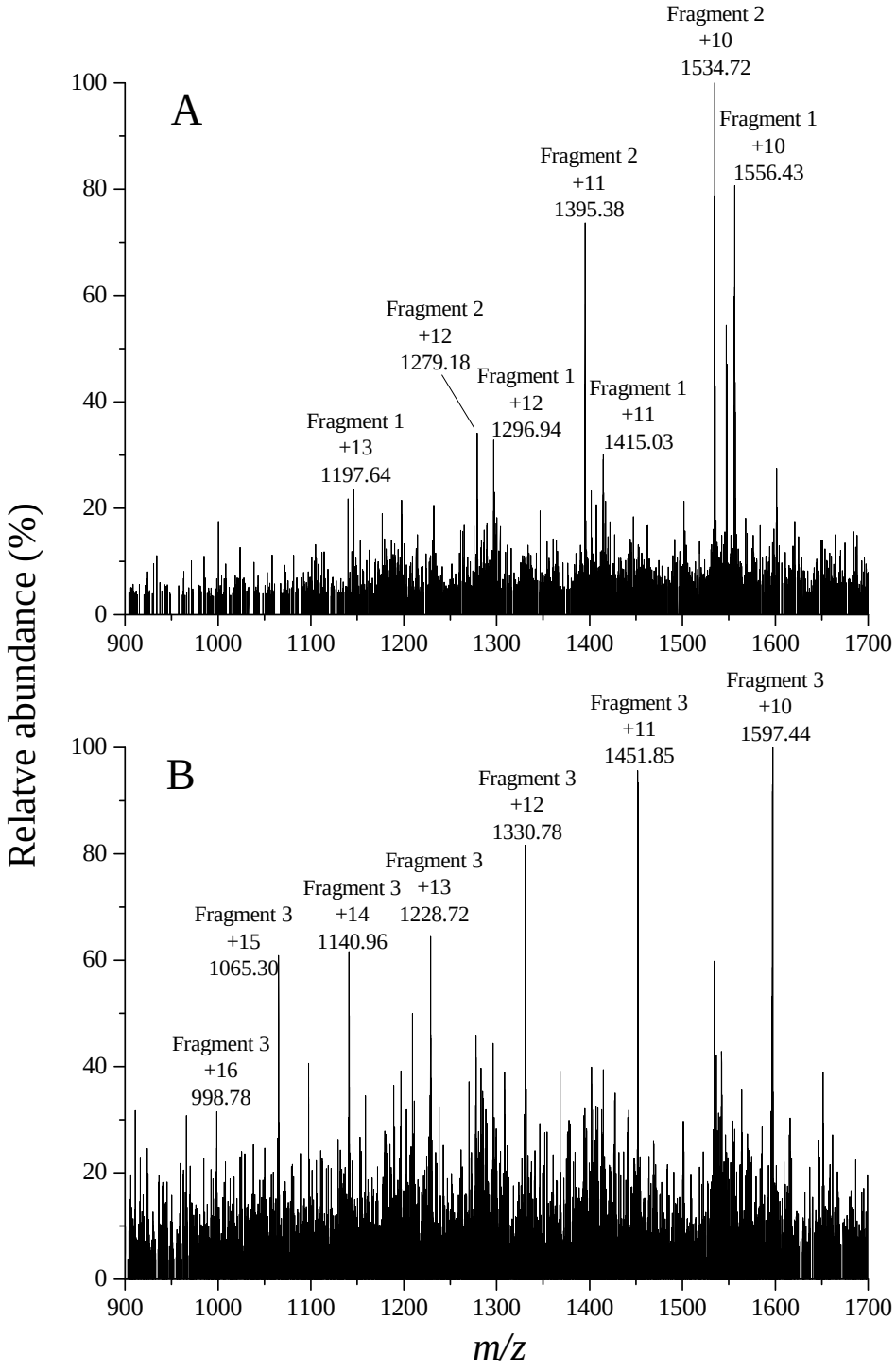
A
Fragment 2
+10
1534.72
Fragment 1
+10
1556.43
Fragment 2
+11
1395.38
Fragment 2
+12
1279.18
Fragment 1
+12
1296.94
Fragment 1
+11
1415.03
Fragment 1
+13
1197.64
B
Fragment 3
+10
1597.44
Fragment 3
+11
1451.85
Fragment 3
+12
1330.78
Fragment 3
+13
1228.72
Fragment 3
+14
1140.96
Fragment 3
+15
1065.30
Fragment 3
+16
998.78
Relatve abundance (%)
m/z

Figure S7

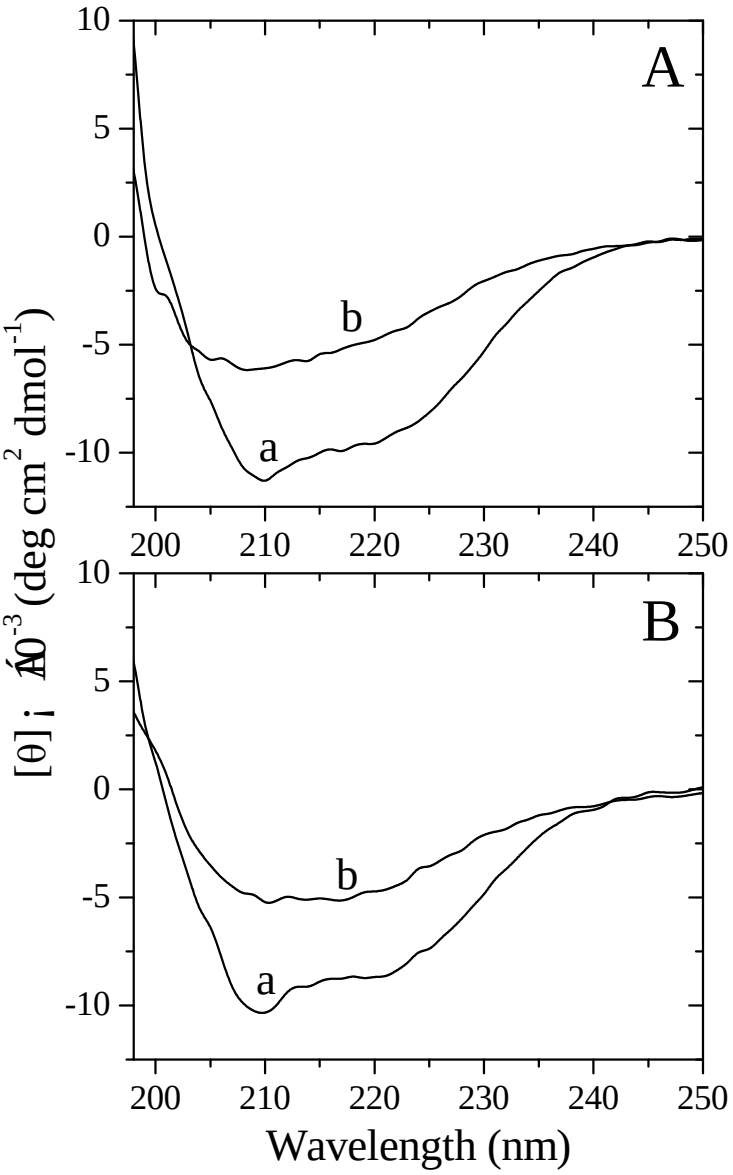

Figure S8

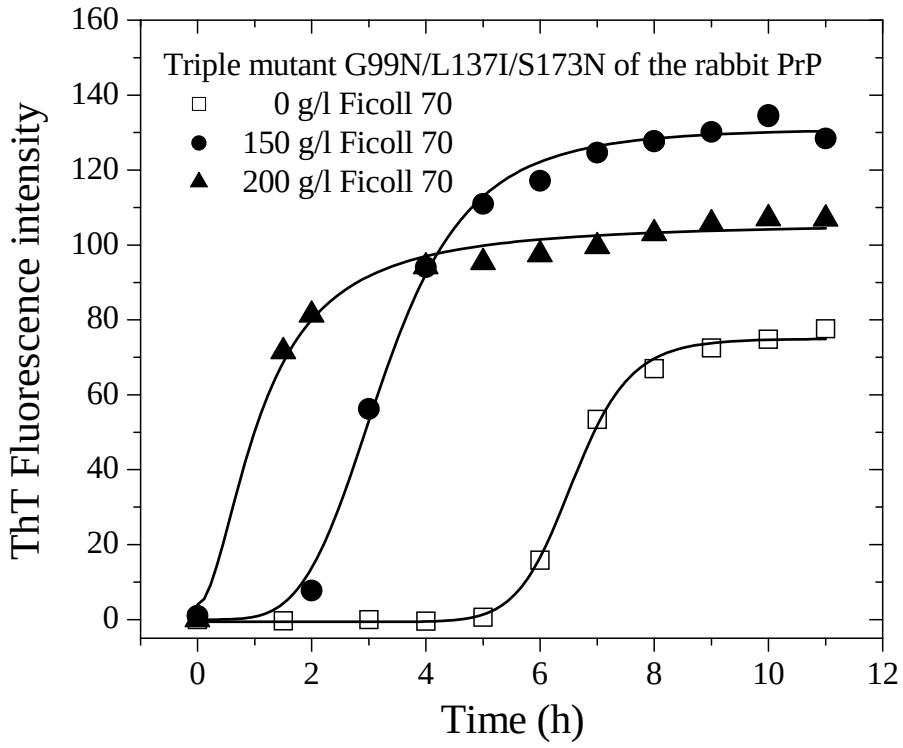
Triple mutant G99N/L137I/S173N of the rabbit PrP
0 g/l Ficoll 70
150 g/l Ficoll 70
200 g/l Ficoll 70
ThT Fluorescence intensity
Time (h)
0
20
40
60
80
100
120
140
160
0
2
4
6
8
10
12

Figure S9