Stability of BSE infectivity towards heat treatment even after proteolytic removal of prion protein

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Abstract
The unconventional infectious agents of transmissible spongiform encephalopathies (TSEs) are prions. Their infectivity co-appears with PrPSc, aberrant depositions of the host’s cellular prion protein (PrPC). Successive heat treatment in the presence of detergent and proteolysis by a keratinase from Bacillus licheniformis PWD-1 was shown before to destroy PrPSc from bovine TSE (BSE) and sheep scrapie diseased brain, however data regarding expected reduction of infectivity were still lacking. Therefore, transgenic Tgbov XV mice which are highly BSE susceptible were used to quantify infectivity before and after the bovine brain treatment procedure. Also four immunochemical analyses were applied to compare the levels of PrPSc. After heating at 115 °C with or without subsequent proteolysis, the original BSE infectivity of 10^6.2–6.4 ID50 g^-1 was reduced to a remaining infectivity of 10^4.6–5.7 ID50 g^-1 while strain characteristics were unaltered, even after precipitation with methanol. Surprisingly, PrPSc depletion was 5–800 times higher than the loss of infectivity. Similar treatment was applied on other prion strains, which were CWD1 in bank voles, 263 K scrapie in hamsters and sheep PG127 scrapie in tg338 ovinized mice. In these strains however, infectivity was already destroyed by heat only. These findings show the unusual heat resistance of BSE and support a role for an additional factor in prion formation as suggested elsewhere when producing prions from PrPC. Leftover material in the remaining PrPSc depleted BSE preparation offers a unique substrate for searching additional elements for prion infectivity and improving our concept about the nature of prions.

Keywords: Prion, PrP, Molecular mechanism, BSE, Zoonotic, Infectivity, Strain, Heat, Inactivation, Bioassay

Introduction
Prions are infectious agents of transmissible spongiform encephalopathies (TSEs) or prion diseases [1]. The infectivity is dependent on a conformationally malformed state (PrPSc) of the physiological protein PrPC, a cellular membrane protein with an as yet unclear function. The mechanism of transformation of this host encoded PrPC to PrPSc includes refolding and aggregation. PrPSc is partially resistant to digestion with proteolytic enzymes, usually proteinase K (PK). During proteolysis—often in the presence of detergent—the PrPSc molecules become N-terminally truncated while the remaining C-terminal part (PrPres) after dissociation and unfolding is characterized by a triplet of a diglycosylated, monoglycosylated and non-glycosylated PrP fragment in the 18–30 kDa molecular mass range.

The proof that PrPSc represents infectivity was first based on biomathematical and extensive biochemical work with hamster scrapie [1–5]. Definitive proof that
the presence of PrPSc is a prerequisite for TSE infection was presented from PrP-less mice, goats and cells, and by the production of infectivity from recombinantly expressed and purified PrP [6–14]. Another argument for the validity of the role of PrP in the agent is the close relation between susceptibility/resistance in e.g. sheep, goats and humans and genetic polymorphisms in the PRNP coding region [15–17]. Yet, the very reproducible strain properties characteristic for TSEs are not yet explained so far. These might be dependent on the presence of lipid, polyanionic glycans or nucleic acid fragments in the agent or during PrPSc formation [9, 13, 18–20]. From observations with bovine spongiform encephalopathy (BSE) inoculated in in-bred wildtype mice it was even postulated that an additional unidentified agent may be essential for transmission while PrPSc would be involved in species adaptation [21].

Previously, we found that B. licheniformis PWD1 keratinase (KE) at 50 °C could reduce PrPSc by more than 99.9% after autoclaving for 40 min at 115 °C in the presence of the detergent sarkosyl at neutral pH [22]. The material used in these experiments was brain stem from cattle and sheep clinically affected respectively by BSE and scrapie. The material after autoclaving for 40 min at 115 °C in the presence of sarkosyl at neutral pH [22]. The material used in these experiments was brain stem from cattle and sheep clinically affected respectively by BSE and scrapie. Other investigators found proteases which already had a substantial PrPSc degrading effect even without heating above 100 °C or the presence of detergents in the homogenate while pH varied between 7–12 and temperatures between 37–70 °C [23–25]. According to these results, there is no direct correlation between PrPSc level and infectivity. This weakens the prion hypothesis which in part is based on a positive correlation between the two parameters [5, 26]. Further confusing are examples of infectivity related to protease sensitive PrPSc [27–29].

In this study, we investigated whether our PrPSc removal from BSE infected cow brain using heating at 115 °C and enzymatic proteolysis goes together with removal of infectivity in the highly sensitive transgenic Tgbov XV mice expressing bovine PrP. The presence of PrPSc was tested in Western blotting and several biochemical methods. We also compared the effect of this brain treating methodology when applied on three other prion isolates with short incubation times respectively 263 K scrapie strain in hamsters, chronic wasting disease (CWD) strain in bank voles and sheep PG127 scrapie in tg338 mice that are expressing sheep PrPVRQ.

Materials and methods

Antibodies

PrP-specific monoclonal antibodies (mAb’s) used were: SAF34, Bar224, 12B2, 9A2, 3F4, 6C2, 12F10, L42, 6H4, Sha31, SAF84, 94B4, F99/97.6.1 [30–37]. Their linear specificities on PrP have been described and further confirmed by Pepscan analysis [38] as follows (bovine PrP numbering, 6 octarepeats): 62QPHGGGW92 (SAF34), 101WGQGG105 (12B2), 110WNK112 (9A2), 117KTNMKHV113 (3F4), 122HVA-GAAA128 (6C2), 152FGSDYEDRYYR162 (Bar224), 154NDYEDRYYREY163 (12F10), 156YEDRYYREY161 (L42), 156YEDRYYRE163 (Sha31), 156YEDRYYREYN164 (6H4), 174YRPVDQY180 (SAF84), 198HTVTTT205 (94B4) and 229YQRE232 (F99/97.6.1).

Proteolytic enzymes

Lyophilized keratinase (KE) was used in purified form (1.4 × 10^4 azocaseine-U mg⁻¹) [39, 40]. Proteinase K (PK) was purchased as lyophilized product (Merck 1.24568; 30 mAnson-U mg⁻¹).

Tissues

Bovine BSE brains were from the rostral part of the obex of a British clinically and histologically confirmed positive BSE cow (UK case 97/0913, kind gift from APHA Weybridge at UK), and obex tissues from confirmed BSE positive Dutch cases NL6 (clinically positive), NL11 and NL19 (clinically healthy at slaughter) as well as from BSE confirmed negative cattle. Hamster brains infected with the 263 K scrapie strain were supplied by RKI Berlin, sheep PG127 scrapie brain isolate was second oral passage material in VRQ/VRQ sheep prepared at ENVT Toulouse, CWD1 isolate was passaged three times in bank voles with PrP genotype 109I/I (Bv109I).

Ethical statement

Animal experimentation was performed in 2004–2009 according to the prevailing regulations of European directives (86/609/EEC) as well as in compliance with the respective national and institutional legislations. The number of animals used were kept at the lowest as considered necessary for the experiments in line with the three R’s concept: replace, reduce and refine. This means per dose group 10–16 animals for Tgbov XV mice, and six for the other rodent bioassays.

Preparation of inocula

Brain materials from cattle and rodents were subjected to similar procedures with disposable equipment. Homogenizations were carried for 45 s at 23 000 rpm in Prypcron vials with a MediFASTH apparatus (Consul AR SA; Villeneuve, Switzerland). Negative tissues were

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1 When in revision, a paper appeared where no PrPSc could be found in one out of three clinically positive steers orally infected with bovine BSE. In this one animal only by PMCA a weak positive signal for PrPSc was found in the 3rd round at highest seeding concentration while Tg BovXV mice remained negative for this steer. The two other steers were clearly PrPSc positive in biochemical tests, immunohistochemistry, PMCA at 1st, 2nd and 3rd round, and were infective in the bovinized mice [59].
first prepared before the positive ones, and dilutions of samples were performed with changing pipet tips for every next dilution step.

Brain stem material from cattle and sheep and rodent brains were homogenized as 10% (w/t) tissue samples either in physiological saline (PS) or in the presence of 2% sarkosyl as detergent in 50 mM sodium phosphate pH 7.5.

The detergent containing homogenate was aliquoted. One aliquot of the heated material was left undigested, and another part was further subjected to digestion for 60 min at 50 °C with 25–50 azocaseine-U KE mg⁻¹ tissue equivalents (TE) unless otherwise stated. For the scrapie and CWD1 experiments an additional impairment in their capacity to feed. Animal brains were of gait, difficulty in rising from a supine position, and such as tremor of head or whole body, incoordination

Animals were culled when positive for clinical signs of PrPres with TeSeE®, SAP combination kit (Bio-Rad). Borderline and negative cases in TgBov XV mice were tested. For further establishing presence of PrPSc or PrPres in BSE related samples, three different EC approved commercial enzyme-linked immunosorbent assays (ELISAs) for BSE testing were carried out: TeSeE® SAP combination kit (Bio-Rad), HerdCheck BSE Ag test (IDEXX Europe BV) and CediTect® BSE test (Prionics Lelystad BV). The general principle of these tests is described in Additional file 1.

Reference internal control samples were exactly treated as in routine testing is required. However, for each of the three tests, study samples were first precipitated with nine volumes of cold methanol by centrifugation for 10 min at 16 000 × g and resuspended in PS. After centrifugation of digested and heated material with or without methanol, a pellet was visible only when methanol was used. The 1% tissue homogenates were heated for 20 min at 80 °C and stored at −80 °C till use.

**Animal studies**

Tgbov XV mice overexpressing bovine PrP that are highly sensitive to bovine BSE infection were used for challenges by intracerebral inoculation with 20 μL of 1% (w/t) and lower doses tissue homogenate [41]. Inocula were prepared (see paragraph below) from the British BSE case. PrPSc detection by Western blotting as previously described [42]. Running buffer was either 3-N-morpholino)propane sulfonic acid (MOPS) or 2-(N-morpholino) ethane sulfonic acid (MES). Staining of protein in gels was performed with the SilverXpress™ kit (Thermo Fisher Scientific) followed by destaining of silver and restaining with Coomassie brilliant blue [46, 47].

Brain material of Tgbov XV mice was tested for PrPres with the TeSeE® purification and detection kit (Bio-rad). End-point titers expressed as 10 log infectious doses per g tissue (ID₅₀ g⁻¹) were determined applying the Spearman-Kaerber method [42].

Syrian hamsters, bank voles (Bv109I) and transgenic mice expressing the sheep PrPVRQ (Tg338) were used respectively for studies with 263 K scrapie infected hamster, CWD1109 from Bv109I after 3rd passage and sheep scrapie isolate PG127. Animal brains were homogenized as 10% (w/t) and lower doses of brain homogenate were intracerebrally inoculated as described [43–45]. In the CediTect BSE test, pellets of the study samples were taken up in the kit specific solution before including in the tests.

In the HerdCheck test, the pellets were resuspended in 600 μL test kit homogenization buffer and incubated at ambient temperature for 10 min. Then, 120 μL was mixed with 30 μL of plate diluent of which 100 μL was added per well of 96 wells IDEXX plate that contains Seproin ligand for binding PrPSc aggregates. Further denaturation to open bound aggregates for antibody binding was performed according to the kit protocol. Per well 0.7 mg TE were tested.

In the CediTect BSE test, pellets of the study samples were resuspended in 100 μL kit lysis buffer and after...
resuspension further ten times diluted with kit lysis buffer of which 50 μL (0.5 mg TE) was applied per well in each of two PVDF filter plates. Further procedure was according to the test protocol which means that after washing with phosphate buffered saline (PBS) by filtration, one plate was treated with PBS and the other with 5 M guanidinium thiocyanate in PBS and the other with PBS only. Per well 0.5 mg TE were tested.

Results

PrP<sup>Sc</sup> digestion in heat treated cattle brain

Heating alone in the presence of 2% sarkosyl at 115 °C of homogenates prepared from brain tissue of a British confirmed BSE cow with clinical signs did not lead to significant loss of PrP<sup>Sc</sup> immunoreactivity (Figure 1B, cfr. lane 4 with lanes 1–3) as was previously also shown [22]. Subsequent digestion with keratinase (KE) at increasing enzyme concentrations showed that all PrP material already disappeared at 5 KE-units per mg tissue equivalents (TE) (Figure 1B, lane 6). Staining with silver and Coomassie brilliant blue both showed that at this low enzyme/tissue ratio proteins were degraded to peptides migrating at 6 kDa and lower and to proteins with molecular masses of 300 kDa and higher (Figure 1 panels A1-2, lanes 6). However only in heated non-digested sample the ≥ 300 kDa fraction was reactive with PrP specific antibodies, but not after digestion with KE (Additional file 2 cfr. lane 4 with lane 7). This means that this large size protein material is accessible for PrP-specific antibodies. Precipitation with methanol and 1-propanol did show that the former treatment yielded acceptable recoveries of both PrP material and other proteins (Figure 1, panel A1, cfr. lane 1 with lanes 2 and 3). The level of PrP-reactive material was roughly

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**Figure 1** Digestion of total protein in BSE infected bovine brain homogenate. Panels A and B were derived from one SDS-PAGE gel, cut into two parts. Similar samples were loaded in the two parts before staining. Staining: A1, total protein with silver; A2, total protein with Coomassie brilliant blue after destaining the silver from A1; B, PrP staining after Western blotting using a mixture of antibodies 9A2 and 94B4 (each at 0.2 μg mL<sup>−1</sup>). Heating in detergent containing homogenization buffer is indicated in the “115 °C” row. KE row shows the azocaseine–U mg<sup>−1</sup> tissue equivalents. Tissue equivalents applied: 250 μg per lane except for lane 4, only 62 μg to prevent overstaining. Samples in lanes 1 and 4–6 not precipitated, in lanes 2 and 3 precipitated respectively with methanol and 1-propanol. Molecular mass markers used were SeeBlue in A and B, and MagicMark XP in C, for which migration positions are indicated in kDa at the left. Gel was run in MES buffer. Top and running front are indicated with arrow heads and arrows, respectively.
the same between non-heated digested (lane 1) and heated non-digested or slightly digested homogenate (lanes 4 and 5).

In heated and KE-digested brain material, the extent of PrPSc removal by keratinase from high-titer central nervous system tissue of the BSE infected cow was above 99.9% (> factor 1000x) by Western blotting with mAb's 98B4 and Sha31 (Figure 2). Other mAb's yielded the same outcome such as SAF34, 9A2, 12B2, 6C2, 12F10, L42, SAF84 and F99/97.6.1. This infers that the destruction of PrP had involved the whole molecule.

Three different ELISA tests were used for quantifying the presence of PrPSc, each using a different property for immobilisation before further analysis, all using PrP specific mAb's and each including a denaturation treatment to enable access of antibodies. These different properties were: 1 in TeSeE test the capture of PrPres by PrP specific antibody coated to polystyrene, 2 in the IDEXX HerdCheck test the binding of PrPSc-fibrils by Sepriion ligand immobilized to polystyrene, and 3 in the Cedi-Tect BSE test the level of unfolding of PrPres—adsorbed to PVDF filters—which reflects its aggregated condition. In these tests PrPSc removal by keratinase in heated samples reached values 99.7–99.96% or alternatively PrPSc/PrPres reduction factors of >2500x, >333x, >500x, and >2500x in respective TeSeE, HerdCheck, and Cedi Tect with 9A2 and 94B4. In fact, each outcome was within borderline background (Table 1).

Taken together, from these three different biochemical analyses using a range of different PrP specific antibodies, it was not possible to show the presence of any leftover PrPSc in the heat-treated, keratinase digested, bovine brain.

Infectivity of BSE material in Tgbov XV mice
Infectivity of untreated cow brain homogenate used in the challenge experiments was estimated at 10^6.2–10^6.4 ID_{50} g^{-1}, a value usual for BSE samples in mice transgenic for bovine PrP (Tgbov XV) [41, 49]. Heating at 115 °C led to an infectivity titer change down to 10^4.6 corresponding to a 40–60 fold titre loss (Table 2). A precipitation step with methanol applied on non-digested samples did increase titers 2- and fivefold for respectively non-heated and heated material which indicated that the precipitation of BSE agent was very effective. Surprisingly, subsequent proteolytic removal of PrPSc did not further remove infectivity, but it rather led to a 12.5-fold increase of infectivity (from 10^4.6 to 10^5.7). For confirmation of these results, we repeated these measurements with newly generated inoculum with dilutions around the critical doses 10^2.5 and 10^3.5. Results agreed with those in the previous experiment: digestion of heat-treated brain homogenate did not additionally remove infectivity but rather increased the infectivity (Figure 3). Thus, here a situation is encountered where removal of all detectable forms of PrP from prions did not further reduce infectivity (Additional file 3).

Moreover, in mice infected with these PrP depleted samples the triple band pattern of PrPres yielded a typical classical BSE strain profile with respect to migration position (e.g. lower band migrating at 19 kDa), a minimal 12B2 reactivity and the diglycosylated fraction...
Infectivity of other TSE materials with short incubation times

To see whether retention of infectivity also would occur in similarly autoclaved TSEs from other sources we chose (upper band) as the major PrP band similar to the sample used for challenge (Figure 4).
rapid infection models which were hamster 263 K scrapie in hamsters, sheep PG127 (or Dawson) scrapie in Tg338 shPrPVRQ mice, and bank vole CWD1109I in 109I/I bank voles (Bv109I) with reported minimal incubation times of less than 100 days. The effect of heat treatment and that of heat treatment plus digestion were checked by Western blotting which confirmed the effectiveness of the PrPSc removal by KE as well as proteinase K (Figure 5). The 10logID50 g−1 titers of non-heated inocula were for 263 K, PG127 scrapie and CWD1109I respectively 6–6.5, 5.8–6.3 and 8.4 (Table 3). However, heat treatment at 115 °C in each of the three systems led already to an infectivity reduction below the detection limit except maybe for bank voles where one out of six animals was positive at highest concentration tested corresponding to a titer of roughly 3.4 10logID50 g−1, which in that case would mean an infectivity reduction of at least 5 10log units. Subsequent digestion with KE or PK of all heated inocula yielded TSE negative outcomes for clinical signs and PrPres testing in Western blots.

These experiments do show that BSE differs from the other three TSEs with respect to resistance of infectivity to heat (i.e. 115 °C for 40 min under wet conditions and in the presence of detergent), a process that allowed proteolytic removal of PrPSc below the detection limits of the tests used.

Discussion

In bovine BSE infected brain homogenate heated under wet conditions at 115 °C for 40 min a high level of infectivity was retained when inoculated in transgenic mice (Tgbov XV mice) expressing bovine PrP. This high level of residual heat resistant infectivity was not further inactivated by exhaustive proteolytic removal of PrPSc. In addition, the molecular BSE-strain type of PrPres appeared conserved in the mice. Similarly treated brain from three other prion sources with short incubation times in rodents lost their infectivity by the heat treatment, confirming the unique heat resistance of the BSE agent from cattle compared to that of other prions.
The loss of titre in bovine BSE brain homogenates after heating at 115 °C in the presence 2% sarkosyl amounted to only 0.7–1.8 \(10^{\log_{10}ID_{50}}\) g\(^{-1}\). Incomplete BSE inactivation tested in Tgbov XV mice compares well with other studies in homogenates using heating at temperatures between 100–140 °C for either bovine BSE in wild type mice and rodent BSE in transgenic mice expressing high levels of murine PrP [49–51]. The infectivity loss of scrapie types 263 K and PG127, and CWD1\(_{1091}\) agrees with studies that BSE carries an exceptional resistance to wet heat.

![Figure 5](image-url)

**Figure 5** Proteolytic digestion of PrP\(^{Sc}\) in brain homogenates infected with PG127 scrapie, 263 K scrapie and CWD1. Heated and digested samples are indicated with an X. Three blots from left to right: sheep PG127, hamster 263 K and bank vole CWD1 immunostained with respectively Sha31, a mix of 3F4 and Sha31, and SAF84. Antibody concentrations 0.5 µg mL\(^{-1}\), except 3F4 at 1.0 µg mL\(^{-1}\). Enz-row shows where proteinase K or keratinase was used for digestion. N in lanes 2, 4, and 6 means TSE negative brain samples. Migration position of molecular mass markers are indicated at the left together with their kDa (SeeBlue markers). Gels were run in MOPS buffer. Tissue equivalents applied: 455 µg mL\(^{-1}\). Per panel lanes 1 and 2–6 are from the same blot.

**Table 3** Infectivity of heat and protease treated whole brain homogenates in the host rodent species

| Infection model | Treatment | Detergent, no heat | Detergent, heat | Detergent, heat, KE | Detergent, heat, PK | NEG Ctrl\(\text{a}\) |
|-----------------|-----------|-------------------|----------------|-------------------|-------------------|-----------------|
| 263 K in hamsters | 6/6 (ID\(_{50}\) = 6.0–6.5) | 0/6 (ID\(_{50}\) < 3.5) | 0/6 (ID\(_{50}\) < 3.5) | 0/6 (ID\(_{50}\) < 3.5) | 0/12 (ID\(_{50}\) < 3.5) |
| | 117 ± 7 d | > 325 d | > 325 d | > 325 d | > 284 d |
| PG127 scrapie in tg338 mice | 6/6 (ID\(_{50}\) = 5.8–6.3) | 0/6 (ID\(_{50}\) < 2.5 ID) | 0/6 (ID\(_{50}\) < 2.5 ID) | 0/6 (ID\(_{50}\) < 2.5 ID) | 0/12 (ID\(_{50}\) < 2.5) |
| | 82 ± 4 d | > 200 d | > 200 d | > 200 d | > 200 d |
| 100\(^{10}\)CWD1 in Bv109I bank voles | 6/6\(^{b}\) (ID\(_{50}\) = 8.4) | 1/6 (ID\(_{50}\) = 3.4) | 0/6 (ID = < 3) | 0/6 (ID = < 3) | 0/12 (ID\(_{50}\) < 3) |
| | 44 ± 4 d | 318 d | > 450 d | > 450 d | > 450 d |

Inocula tested at 1% tissue concentration, except where indicated. Titres were based on survival times and deduced for hamster 263 K scrapie as in [1], for sheep PG127 scrapie from end-point titration curve in [43], and for CWD1\(_{1091}\) from end-point titration curve in [42].

\(a\) 12 animals, three in each of the four treatments.

\(b\) Unheated material tested with 0.1% inoculum; other treatments with bank voles were in addition to 1% also checked at 0.1 and 0.01% tissue concentrations and all with no attacks as result.
heat conditions compared to many other TSE strains that get largely inactivated already below 100 °C [49, 52–55].

Remarkably, removal of PrPSc with keratinase after heating at 115 °C from BSE and other TSE strains did confirm the effectivity of the enzymatic digestion with keratinase (and proteinase K) when tested in Western blotting and, in case of BSE, in commercially available diagnostic tests. It may be that in the BSE material after heating and keratinase digestion some form(s) of PrPSc were preserved and remained undetectable. Protein material was found in both low molecular mass region < 6 kDa and in the > 300 kDa protein fraction, but both were not immunoreactive with PrP-specific antibodies. Also, additional testing for the presence of PrP using three different sensitive biochemical diagnostic ELISAs did not reveal residual signs of PrPSc. Furthermore, an extra dissociation and unfolding treatment on Western blot PVDF membranes with guanidinium thiocyanate also did not lead to any binding of PrP specific antibody (not shown). If sub-background amounts of PrPSc or fragments thereof still were present in the inoculum, then the difference between infectivity reduction by heat (3–60 fold decrease) and PrPSc by subsequent breakdown with keratinase (333–2500 fold decrease) does not well correlate since these differences between the bioassay and biochemical data range between 5–800 fold. This was also observed by other studies with BSE, where infectivity and PrP-immunoreactivity cannot be simply compared in contrast to prion seeding assays like protein misfolding cyclic amplification (PMCA) and infectivity testing [49, 56]. If PrP gets fully removed by our method—which is difficult to prove by the relative limited sensitivity of antibodies compared to infectivity testing in transgenic mice—other molecular entities should still be present that attribute to the PrPC to PrPSc conversion. Nevertheless, the discrepancy between infectivity and PrPSc reduction in our study and another study [24] is quite large which justifies searching for alternative factors or cofactors that promote PrPSc propagation depending on strain and environment. In fact, molecules like phospholipid, dextran sulfate and RNA have been reported to be involved in in vitro PrPSc prion formation and infectivity [4, 9, 18, 20, 49]. In this respect BSE represents a unique example of a prion agent that it is able to transmit to many other species and to induce PrP-PrPSc conversion within one species irrespective PrP-polymorphisms [57, 58].

The bovine BSE-typical molecular PrP triple band profile and molecular masses were retained in the brains of transgenic mice expressing bovine PrP when inoculated with heat and keratinase treated bovine BSE brain material. Also, the clinical signs observed were similar in the different inocula used. This corroborates observations about thermostability of strains and the hypothesis that the prion agent contains both a host (i.e. PrP) and a strain dependent component, the latter of which could be a non-protein component [24, 54].

Infectivity was not tested in KE-digested BSE samples that were not heated since we were focused on preparations where PrP was absent as an opportunity to produce a process for significant removal of BSE infectivity. Such test could have yielded information whether in case of BSE a proteolytic digestion of non-heated prion material had lead to infectivity reduction as is the case for e.g. with purified hamster scrapie material [5]. Nevertheless, we did test for the presence of PrPSc in such samples by Western blots where the level of PrP-reactive material did hardly differ between non-heated digested and heated non-digested or partly-digested material, which suggests that infectivity also would have been retained in the non-heated material after digestion.

An approximately tenfold titre increase of infectivity was observed after keratinase treatment of the heated BSE samples which appeared reproducible in a second experiment (from 10^{4.6} to 10^{5.7} ID_{50} g^{-1}, Table 2 and Figure 3). This increase leads us to some assumptions. Possibly there was still an undetectable amount of PrPSc present that under the conditions of the proteolytic treatment attained an increased PrP seeding capacity. Another more complicated possibility would be that after heating a strain determining factor for prion formation was released in the brain homogenate by the protease used. In that case, new PrPSc could be generated from PrP in the transgenic mouse brain leading to de novo infectivity while BSE strain properties remained the same. This latter situation also requires a complete conversion process leading to an infectivity titre higher than before proteolysis. The factor most probably is a macromolecular product still present in the pellet after methanol treatment.

The protein only theory has allowed to better understand the nature and origin of prion agents with PrPSc as the carrier of infectivity. As example of the validity of the prion hypothesis is the application of the one gene—one protein concept in the successful Mendelian way of breeding for resistance towards the disappearance of scrapie in sheep and observation of increased levels of a resistance related polymorphism in humans in the epidemic kuru region in Papua New Guinea [15, 16]. Yet, explaining the molecular basis of strains with their phenotypical behavior based on a polymorphic appearance of PrPSc remains a challenge. Here, the exceptional resistance of BSE to heat and the subsequent removal of PrPSc makes this prion type a rather unique substrate for solving these prion strain questions. The answers could be found in the precipitable leftover material of the heated and digested BSE
infected whole brain preparations. Thus, varying heat treatment and the enzymatic digestion conditions such as pH, detergent and choice of protease could well be of use in figuring out which additional molecular fraction can modulate the PrPSc to PrPres conversion towards strain related properties. Maybe other heat resistant strains than BSE could serve this aim with the potential practical advantage of absence of zoonotic behavior.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13567-021-00928-8.

Additional file 1. Principle of the three ELISA tests to detect bovine PrPSc and PrPSc.

Additional file 2. PrPSc digestion by keratinase of bovine brain after heat treatment at 115 °C. The infected brain in lanes 4, 7 and 10-13, negative control brain in lanes 2 and 3. Material in lanes 2 and 4 was heated at 115 °C in presence of detergent before digestion. Lanes 3 and 10-13: non-heated material digested by KE. In the heated BSE sample in lane 4 no PrP-specific immunoreactivity has remained neither throughout the lane nor in the high molecular mass region at the top, while in the non-heated material there was (lane 10). Lanes 1 and 9, mixture of molecular mass markers SeeBlue and MagicMark XP for which migration positions are indicated in kDa at the left; lanes 5, 6 and 8, no sample applied. Top and running front are indicated with arrow heads and arrows, respectively.

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Authors’ contributions
JPML performed all TSE-inactivations and the initial biochemical analyses; AB-B and MHG contributed with Tgbov XV mice design, Wblot screening and immunohistochemistry; MB performed Tgbov mice experimental work; RO, LP and UA carried out bank vole challenges and analyses; AT and MB performed hamster challenges and analyses; OA carried out tg338 mice challenges and analyses; AD performed ELISA analyses on bovine BSE materials; JS initiated, provided keratinase, and directed the BSE work. All authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare that they have no competing interests.

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References
1. Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. Science 216:136–144
2. Brown P, Liberki PP, Wolf P, Gajdusek DC (1990) Conservation of infectivity in purified fibrillary extracts of scrapie-injected hamster brain after sequential enzymatic digestion or polyacrylamide gel electrophoresis. Proc Natl Acad Sci USA 87:7240–7244
3. Griffith JS (1967) Self-replication and scrapie. Nature 215:1043–1044
4. Ma J, Wang F (2014) Prion disease and the “protein-only hypothesis.” Essays Biochem 56:181–191
5. McKinley MP, Bolton DC, Prusiner SB (1983) A protease-resistant protein is a structural component of the scrapie prion. Cell 35:57–62
6. Avar M, Heinker D, Steineke N, Dogancay B, Moos R, Lugan S, Cosenza C, Hornemann S, Andreoletti O, Aguzzi A (2020) Prion infection, transmisson, and cytopathology modeled in a low-biohazard human cell line. Life Sci Alliance 3:e202000814
7. Bueller H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, Weissmann C (1993) Mice devoid of PrP are resistant to scrapie. Cell 73:1339–1347
8. Deleaut NR, Harris BT, Rees JR, Suppatapon S (2007) Formation of native prions from minimal components in vitro. Proc Natl Acad Sci U S A 104:9741–9746
9. Fernandez-Borges N, Di Bari MA, Erana H, Sanchez-Martin M, Pirisun L, Parra B, Elezagari SR, Vanni I, Lopez-Moreno R, Vaccari G, Venegas V, Charco JM, Gil D, Harrahi C, D’Agostino C, Agrimi U, Mayoral T, Requena JR, Nonno R, Castilla J (2018) Cofactors influence the biological properties of infectious recombinant prions. Acta Neuropathol 135:179–199
10. Kim JI, Cali I, Surewicz K, Kong Q, Raymond GJ, Atarashi R, Qing L, Lambrecht BN, Gander CM, Tang L, Parra B, Elezgarai SR, Vaccari G, Venegas V, Charco JM, Gil D, Harrahi C, D’Agostino C, Agrimi U, Mayoral T, Requena JR, Nonno R, Castilla J (2018) Cofactors influence the biological properties of infectious recombinant prions. Acta Neuropathol 135:179–199
11. Makarava N, Kovacs GG, Bocharova O, Savchenko R, Alexeeva I, Budka H, Rohwer RG, Baskakov IV (2010) Recombinant prion protein induces a new transmissible prion disease in wild-type animals. Acta Neuropathol 119:177–187
12. Salvesen O, Espenes A, Reiten MR, Vuoengo TT, Malachin G, Tran L, Andreoletti O, Olsaker I, Benestad SI, Tranulis MA, Erdelc C (2020) Goats naturally devoid of PrPSc are resistant to scrapie. Vet Res 51:1
13. Wang F, Wang X, Yuan CG, Ma J (2020) Generating a prion with bacterially expressed prion protein in the absence of any mammalian cofactors. J Biol Chem 285:14083–14087
14. Watts JC, Giles K, Stohr J, Oehler A, Bhardwaj S, Grillo SK, Patel S, DeArmond SJ, Prusiner SB (2012) Spontaneous generation of rapidly transmissible prions in transgenic mice expressing wild-type bank vole prion protein. Proc Natl Acad Sci USA 109:3498–3503
15. Hagaengnas TJ, Melchor MB, Windig JJ, Bossers A, Davidek A, van Zijderveld FG (2018) Modelling of strategies for genetic control of scrapie in sheep: the importance of population structure. PLoS One 13:e0195009
16. Mead S, Whitfield J, Poulter M, Shah P, Uphill J, Campbell T, Al-Dujaily H, Escamez P, Girones R, Herman L, Koutsoumanis K, Lindquist R, Nunnung B, Robertson L, Lu G, Sanha M, Skandarins P, Speybroeck N, Simmons M, Ter Kuile B, Thyfress J, Wahlstrom H, Acusti P-L, Andreoletti O, Goldmann M, Langeveld J, Windig JJ, Ortiz Pelaez A, Saney E (2017) Genetic resistance to transmissible spongiform encephalopathies (TSE) in goats. EFSA J 15:4962
18. Deleauft NR, Walsh DJ, Piro JR, Wang F, Wang X, Ma J, Rees jrR, Supat‐
tapon S (2012) Cofactor molecules maintain infectious conformation and restrict strain properties in purified pros. Proc Natl Acad Sci U S A 109:1938–1944.
19. Hunter GD, Gibbons RA, Kimberlin RH, Millson GC (1969) Further studies of the infectivity and stability of extracts and homogenates derived from scrapie affected mouse brains. J Comp Pathol 79:101–108
20. Simonneau S, Thomzig A, Ruchoux MM, Vignier N, Daus ML, Poleggi A, Lebon P, Freire D, Durand V, Graziano S, Galeno S, Galdo F, Cardone F, Comoy E, Pocchian M, Beekes M, Deslys JP, Fournier JG (2015) Synthetic scrapie infectivity: interaction between recombinant PrPc and scrapie brain‐
derived RNA. Virulence 6:132–144
21. Lasmezas CJ, Deslys JP, Robain O, Jaegly A, Beringue V, Peyrin J, Fournier
22. Langeveld JP, Wang JI, Van de Wiel DF, Shi GC, Garsenia GA, Bosser A, Shi J, (2003) Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. J Infect Dis 188:1782–1789
23. Dickinson J, Murdoch H, Dennis MJ, Hall GA, Bott R, Crabb WD, Penet C, Sutton JM, Raven ND (2009) Decontamination of prion protein (BSE301V) using a genetically engineered protease. J Hosp Infect 72:65–70
24. Miyazawa K, Emmerling K, Manuelidis L (2011) High Cj density infectivity remains after prion protein is destroyed. J Cell Biochem 112:3630–3637
25. Sklaviadis TK, Manuelidis L, Manuelidis EE (1989) Physical properties of the Creutzfeldt-Jakob disease agent. J Virol 63:1212–1222
26. Silveira JR, Raymond GI, Hughson AG, Race RE, Smith VL, Hayes SF, Crabb WD (2005) The most infectious prion protein particles. Nature 437:257–261
27. Colby DW, Prusiner SB (2011) Prions. In: Mombrot R, Kelly J, Selkoe D (eds). Cold Spring Harbor Perspect Biol pp 1–23
28. Connor S, Gros N, Tattum MH, Jackson GS, Clarke AR, Collinge J, Wadsworth JP (2008) Detection and characterization of proteinase K‐sensitive disease‐related prion protein with thermolysin. Biochem J 416:297–305
29. Leske H, Herrnemann S, Herrmann US, Zh C, Dammert P, Liu B, Laferriere F, Polymeniou M, Pelzar P, Reimann RR, Schwar P, Rushing EJ, Wuthrich K, Aguzzi A (2017) Protease resistance of infectious prions is suppressed by removal of a single atom in the cellular prion protein. PLoS One 12:e0170503
30. Demart S, Fournier JG, Creminon C, Frobert Y, Lamoury F, Marce D, Lasmezas C, Dormond D, Gras J, Deslys JP (1999) New insight into abnormal prion protein using monoclonal antibodies. Biochem Biophys Res Commun 265:652–657
31. Feraudet C, Morel N, Simon S, Volland H, Frobert Y, Creminon C, Villette D, Lehmans S, Grass J (2005) Screening of 145 anti‐PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. J Biol Chem 280:11247–11258
32. Harmeyer S, Pfaff E, Gruschen MH (1989) Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants. J Gen Virol 70:937–945
33. Jacobs JG, Bosser A, Rezaei H, van Keulen LJ, McCutcheon S, Sklav‐
aidis T, Lantier I, Berthon P, Lantier F, van Zijderveld FG, Langeveld JP (2011) Proteinase K‐resistant material in ARR/VRQ sheep brain affected with classical scrapie is composed mainly of VRQ prion protein. J Virol 85:12537–12546
34. Jacobs JG, Langeveld JP, Biacone AG, Acutis PL, Polak MP, Gavier‐Widen D, Buschmann A, Caramelli M, Casalone C, Maaza M, Gronbach M, Erkens JH, Davide A, van Zijderveld FG, Barou T (2007) Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe. J Clin Microbiol 45:1821–1829
35. Kascak RJ, Rubenstein R, Minza Y, Tonna‐DeMax J, Mersko F, Sier PK, Rabin B, Wsienski HM, Diringer H (1987) Mouse polyclonal and monoclonal antibody to scrapie‐associated fibril proteins. J Virol 61:3688–3693
36. O’Rourke KJ, Badner TV, Besser TE, Miller JM, Culp FC, Wells GA, Ryder SJ, Parish SM, Hamir AN, Rees JA, Knobels DP (2000) Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue. J Clin Microbiol 38:3254–3259
37. Thuring CM, van Keulen LJ, Langeveld JP, Womans ME, van Zijlverd FO, Sweeney TC (2005) Immunohistochemical distinction between preclinical bovine spongiform encephalopathy and scrapie in sheep. J Comp Pathol 132:59–69
38. Slootsma JW, Pujic WC, Ligtvoet GJ, Langeveld JP, Meulen RH (1996) Structural aspects of antibody‐antigen interaction revealed through small random peptide libraries. Mol Membrane Biol 13:87–96
39. Lin X, Lee CG, Casale ES, Shih JC (1992) Purification and characterization of a keratinase from a feather‐degrading Bacillus licheniformis strain. Appl Environ Microbiol 58:3271–3275
40. Wang J, Shi J, (1999) Fermentation production of keratinase from Bacillus licheniformis PWD-1 and a recombinant b subtilis FDB-29. J Ind Biotechnol Biotechnol 22:608–616
41. Buschmann A, Gruschen MH (2005) Highly bovine spongiform encephalopathy‐sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle. J Infect Dis 192:934–942
42. Hubert JJ (1992) Bioassay. Kendall/Hunt Publishing Co, USA
43. Andreoletti O, Orge L, Benestad SL, Beringue V, Litaise C, Simon S, Le D, Aurela D, Simmons H, Lavan C, Corbierie F, Costes P, Morel N, Schelcher F, Lacroix C (2011) Atypical/Nov98 scrapie infectivity in sheep peripheral tissues. PLoS Pathog 7:e1001285
44. Beekes M, Baldauf D, Diringer H (1996) Sequential appearance and accumulation of pathognomonic markers in the central nervous system of hamsters orally infected with scrapie. J Gen Virol 77:1925–1934
45. Di Barri MA, Nonno R, Castilla J, D’Agostino C, Pirisano L, Riccardi G, Conte M, Richt J, Kunke L, Langeveld R, Vaccari G, Agrimi U (2013) Chronic wasting disease in bank voles: characterisation of the shortest incubation time model for prion disease. PLoS Pathog 9:e100321
46. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
47. Switzer RC 3rd, Merril CR, Shifrin S (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal Biochem 98:231–237
48. Thormig A, Kranz L, Giersch G, Kruger D, Beekes M (2003) Widespread PrPSc accumulation in muscles of hamsters orally infected with scrapie. EMBO Rep 4:530–533
49. Marin-Moreno A, Aguilar-Calvo P, Moudou M, Espinoza JC, Beringue V, Torres JM (2019) Thermostability as a highly dependent prion strain feature. Sci Rep 9:11396
50. Schreuder BE, Geertser RE, van Keulen LJ, van Asten JA, Enthoven P, Oberthur RC, de Koejer J, Oosterhaus AD (1998) Studies on the efficacy of hyperbaric rendering procedures in inactivating bovine spongiform encephalopathy (BSE) and scrapie agents. Vet Rec 142:474–480
51. Taylor DM, Fraser H, McConnell L, Brown DA, Brown KL, Lamza KA, Smith GR (1994) Decontamination studies with the agents of bovine spongi‐form encephalopathy and scrapie. Arch Virol 139:313–326
52. Ferrini K, Steele PJ, Taylor DM, Somervelle RA (2007) Comparative studies on the thermostability of five strains of transmissible spongiform encephalopathy agent. Biotechnol Appl Biochem 47:175–183
53. Matsuura Y, Ishikawa Y, Murayama Y, Yokoyama T, Somervelle RA, Kitamoto T, Mohri S (2020) Eliminating transmissibility of bovine spongiform encephalopathy by dry‐heat treatment. J Gen Virol 101:136–142
54. Somervelle RA, Gentles N (2011) Characterization of the effect of heat on agent strains of the transmissible spongiform encephalopathies. J Gen Virol 92:1738–1748
55. Taylor DM, Ferrini K, Steele PJ, McConnell L, Somervelle RA (2002) Thermo‐stability of mouse‐passaged BSE and scrapie is independent of host PrP genotype: implications for the nature of the causal agents. J Gen Virol 83:3199–3204
56. Ackermann I, Shawwalu J, Keller M, Fatola OI, Gruschen MH, Balkema‐Buschmann A (2018) Exploring PMCA as a potential in‐vitro alternative method to mouse biossays for the highly sensitive detection of BSE prions. Berl Münch Tierärztl Wochenschr O/ 131:9/10, DOI https://doi.org/10.2376/0005-9366-18021
57. Priem J, Langeveld JP, van Keulen LJ, van Zijderveld FG, Andreoletti O, Bossers A (2014) Enhanced virulence of sheep-passaged bovine spongiform encephalopathy agent is revealed by decreased polymorphism barriers in prion protein conversion studies. J Virol 88:2903–2912
58. Torres JM, Espinosa JC, Aguilar-Calvo P, Herva ME, Relano-Gines A, Villa-Diaz A, Morales M, Parra B, Alamillo E, Brun A, Castilla J, Molina S, Hawkins SA, Andreoletti O (2014) Elements modulating the prion species barrier and its passage consequences. PLoS One 9:e89722
59. Dudas S, Anderson R, Staskevicus A, Mitchell G, Cross JC, Czub S (2021) Exploration of genetic factors resulting in abnormal disease in cattle experimentally challenged with bovine spongiform encephalopathy. Prion 15:1–11

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