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Quantitative analysis of wet-heat inactivation in bovine spongiform encephalopathy

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ABSTRACT

The bovine spongiform encephalopathy (BSE) agent is resistant to conventional microbial inactivation procedures and thus threatens the safety of cattle products and by-products. To obtain information necessary to assess BSE inactivation, we performed quantitative analysis of wet-heat inactivation of infectivity in BSE-infected cattle spinal cords. Using a highly sensitive bioassay, we found that infectivity in BSE cattle macerates fell with increase in temperatures from 133 °C to 150 °C and was not detected in the samples subjected to temperatures above 155 °C. In dry cattle tissues, infectivity was detected even at 170 °C. Thus, BSE infectivity reduces with increase in wet-heat temperatures but is less affected when tissues are dehydrated prior to the wet-heat treatment. The results of the quantitative protein misfolding cyclic amplification assay also demonstrated that the level of the protease-resistant prion protein fell below the bioassay detection limit by wet-heat at 155 °C and higher and could help assess BSE inactivation. Our results show that BSE infectivity is strongly resistant to wet-heat inactivation and that it is necessary to pay attention to BSE decontamination in recycled cattle by-products.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal and progressive neurodegenerative diseases that include bovine spongiform encephalopathy (BSE), Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep and goats, and chronic wasting disease in deer and elk. In TSE-affected animals, abnormal prion proteins (PrP^{Sc}), a disease-related isoform of a host-encoded normal prion protein (PrP^{C}), accumulates in the brain and is partially resistant to proteinase K digestion. According to the prion hypothesis, PrP^{Sc} may be the major component of the TSE infectious agent (also known as prions) [1,2] and mediate the structural conversion of PrP^{C} into PrP^{Sc}. Experimentally, TSE seeding activity that involves this conversion is monitored by the protein misfolding cyclic amplification (PMCA) assay [3].

TSE agents are highly resistant to conventional physical and chemical procedures for inactivating or eliminating microbes. Inactivation efficiency of the agents changes depending on the tissue states (homogenate or macerate), type of heating (wet or dry) or autoclave mechanism (gravity displacement or porous-load) [4]. In addition, the degree of inactivation varies between TSE agent strains [5]. Several papers have reported that the BSE agent causing variant CJD is more resistant to wet-heat inactivation than other agents of CJD and scrapie [6–9] and survives even after treatment at 134 °C for up to 2 h [8, 9]. Incomplete inactivation of BSE infectivity in cattle tissues during rendering was probably one of the major contributory causes of the BSE epizootic. Accordingly the inactivation profile of BSE infectivity in cattle tissues provides important information for assessment of measures for the control and prevention of the disease.

This report describes a quantitative analysis of BSE infectivity in cattle tissues after wet-heat treatment in a gravity displacement autoclave. We investigated the effect of tissue dehydration on wet-heat inactivation by testing wet-heat treatment on BSE cattle tissues with differing water content. After wet-heat treatment of BSE cattle tissues, the infectivity was quantitatively evaluated using a bioassay involving bovinized transgenic mice. We then confirmed the degree of BSE inactivation using the quantitative PMCA assay [10, 11].

2. Materials and methods

2.1. BSE-infected cattle tissue

The spinal cords from 4 cows (codes 3217, 4612, 5087, and 5523) experimentally inoculated with classical BSE [12] were pooled and homogenized without any additional fluid using a

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blender. The material was divided into small aliquots (200 ± 5 mg) and put on glass dishes (10 × 10 mm; diameter × height). Samples of fully hydrated macerate were used without further processing. Semi-dried macerate was obtained by exposing samples to a dry-air oven at 65 °C for 2 h, resulting in a 30% weight loss. Fully dried macerates were obtained by exposing samples to 65 °C for 24 h in the dry air oven, resulting in 70% weight loss. The BSE infectivity of one macerate sample was measured by the end point titration assay in bovinized transgenic mice (Table 1).

2.2. Wet-heat treatment of samples

Samples placed in glassware were directly transferred to a container (5 mL volume) and autoclaved for 20 min in a TEM-D300M system (Taiatsu Techno Corporation, Tokyo, Japan) as previously described [13]. Temperature and pressure were monitored during wet-heat treatment. The exposure time was counted from the point the target temperature ranged from 133 °C to 170 °C (see Table 2). Treatments were performed in duplicate or triplicate. After wet-heat treatment, the sample was transferred to a new tube and homogenized in 10% (w/v) phosphate-buffered saline (PBS), based on the initial macerate weight. All homogenates were stored at −80 °C and sonicated prior to analysis.

2.3. Bioassay in bovinized transgenic mice

The bovinized transgenic mouse Ki+Tg#40, carrying the open reading frame of bovine PrP with 6 octarepeats, is homozygous for both transgenes of knock-in and random insertion, and expresses 12-fold more bovine PrP^C in the brain than a bovinized knock-in mouse but does not express mouse PrP^C (data not shown) [14]. The mice were intracerebrally inoculated with 20 μL of 10% homogenates of the treated and untreated samples. To calculate the 50% lethal dose in 1 g equivalent cattle tissue (LD_{50}/g), the same volumes of 10-fold dilutions of untreated BSE macerate were inoculated into the right hemisphere. The bioassay also yielded a standard curve for the relationship between incubation period and LD_{50}/g. The mice were housed in a biosafety level 3 room of our animal facility and were monitored every 2 d; they were euthanized once they fell ill or were observed until 850 d post inoculation. The incubation period (in days) from inoculation to euthanasia has been provided as the mean and standard deviation value for BSE-affected mice in each group.

To confirm BSE transmission, the brains of the euthanized mice were subjected to histopathology analysis [15] and Western blotting with the anti-PrP monoclonal antibody 6H4 (Prionics AG, Switzerland). The mouse brain was sliced in half sagittally; the right brain was fixed in formalin for histopathology analysis and the other half was frozen at −80 °C for Western blot analysis. BSE-affected mice were identified by immunohistochemical analysis of PrP^{Sc} deposits in the mouse brain or proteinase K resistant PrP^{Sc} on Western blots.

Table 1
End-point titration assay of untreated BSE macerate.

Dilution factor	n/n_0[a]	Incubation period (mean days ± SD)
−1[b]	7/7	321 ± 26
−2	6/6	326 ± 30
−3	5/5	384 ± 17
−4	5/6	424 ± 66
−5	0/6	–[c]
−6	0/6	–

[a] Number of BSE-affected mice per population of inoculated mice.
[b] 10% homogenate of the BSE macerate.
[c] Mice who did not have BSE until 850 d post inoculation.

All animal experiments were approved by the Committee of Animal Experiment, National Institute of Animal Health, and were performed according to the Guideline for Animal Experiment of the Ministry of Agriculture, Forestry, and Fisheries of Japan.

2.4. Quantitative PMCA

The treated and untreated 10% cattle macerate homogenate inocula were considered −1 log dilution and were then serially diluted (10-fold). The diluted samples (2 μL) with 78 μL of PrP^C substrate containing 0.5% potassium dextran sulfate were subjected to PMCA with 40 cycles of sonication (one pulse oscillation for 5 s, repeated five times at 1 s intervals), followed by incubation at 37 °C for 1 h with agitation [11]. The PMCA product (16 μL) with 64 μL of the substrate was used to initiate each subsequent round, and the process was repeated for a total of 4 rounds. The PMCA products were assayed by Western blotting for the detection of proteinase K-resistant PrP^{Sc}. The assays were performed in quadruplicate and were calculated as the log dose of 50% available PMCA seeding activity per 1 g wet weight equivalent (indicated as log $PMCA_{50}$/g) by the Spearman–Kärber method, using the positive and negative seeding activity ratios in each 10-fold dilution.

3. Results

3.1. Infectivity titer of the untreated BSE macerate

Ki+Tg#40 mice developed mouse BSE at 321 d post inoculation with 10% untreated BSE homogenate. The incubation period was prolonged and the rate of mice affected with BSE decreased with increasing dilution ratios (Table 1). The infectivity detection limit was −4 log dilution of the untreated BSE macerate. The infectivity titer of the untreated BSE macerate was calculated as 6.03-log LD_{50}/g by the Spearman–Kärber method by using the ratios of positive and negative mice in each dilution group.

3.2. Bioassay of BSE cattle tissues using bovinized transgenic mice

Ki+Tg#40 mice were affected with BSE when inoculated with macerate treated at 133–150 °C. The incubation periods increased with increase in processing temperature (Table 2). No mice were infected after inoculation with macerate heated at or above 155 °C. Mice inoculated with BSE semidry materials that were treated at 133–155 °C were affected with BSE, but inocula treated at ⩾160 °C did not produce disease. The incubation periods increased with increase in processing temperature. In the case of dry BSE materials, mice were affected with BSE even at 170 °C in 1 of the 3 samples tested. The incubation periods were occasionally shorter than those for untreated material, with processing temperatures from 133 °C to 155 °C. No significant histological differences between infected brains were observed (data not shown).

As shown in **Fig 1**, the available data indicate that BSE infectivity was stable until near a temperature of 130 °C, but then in rapidly reduced with increasing temperature. Dried material was not substantially inactivated until a temperature in excess of 150 °C was obtained, but higher temperatures rapidly reduced infectivity to undetectable levels above 170 °C. Infectivity titers were calculated from incubation periods using the dose response curve obtained from the titration of the macerate (6.03-log LD_{50}/g) (Table 1). The calculated titers for macerate heated at 133 °C ranged from 4.03-log LD_{50}/g and 4.70-log LD_{50}/g, and fell to undetecatable levels at 155 °C. Infectivity in treated semidry material also decreased with increasing temperature, compared to 5.03-log LD_{50}/g in the untreated semidry tissue, and reached the detection limit at 155 °C. In the case of BSE dry materials, infectivity after

Table 2
Infectivity in BSE tissues after wet-heat treatment.

Temperature (Pressure)[a]	Macerate		Semidry		Dry	
	n/n_0[b]	incubation period (mean ± SD)	n/n_0[b]	incubation period (mean ± SD)	n/n_0[b]	incubation period (mean ± SD)
No treatment	7/7	321 ± 26	8/8	326 ± 41	5/5	319 ± 36
133 °C (0.21 MPa)	6/6	384 ± 43	6/6	353 ± 28	6/6	337 ± 20
	5/5	345 ± 19	5/5	325 ± 14	4/4	302 ± 13
	6/6	359 ± 26	6/6	339 ± 54	5/5	287 ± 6
136 °C (0.22 MPa)	6/6	363 ± 39	6/6	349 ± 31	5/5	313 ± 19
	5/5	430 ± 61	5/5	385 ± 36	6/6	306 ± 18
	6/6	388 ± 62	6/6	343 ± 55	6/6	294 ± 12
140 °C (0.28 MPa)	5/5	417 ± 31	6/6	374 ± 38	5/5	343 ± 41
	5/5	406 ± 35	5/5	444 ± 70	6/6	293 ± 17
145 °C (0.32 MPa)	4/5	555 ± 64	6/6	426 ± 71	5/5	294 ± 37
	6/6	465 ± 33	5/5	422 ± 35	6/6	301 ± 27
	5/5	476 ± 91	5/5	391 ± 45	6/6	343 ± 36
150 °C (0.38 MPa)	4/6	536 ± 51	4/4	483 ± 60	5/5	312 ± 43
	3/6	608 ± 72	5/5	503 ± 21	5/5	291 ± 9
155 °C (0.44 MPa)	0/6	–[c]	2/5	474 ± 144	5/5	309 ± 55
	0/6	–	4/6	583 ± 39	6/6	334 ± 50
	0/5	–	0/5	–	5/5	363 ± 55
160 °C (0.52 MPa)	0/4	–	0/4	–	6/6	350 ± 46
			0/6	–	5/5	335 ± 42
165 °C (0.60 MPa)	0/6	–	0/5	–	3/5	467 ± 99
	0/6	–	0/5	–	1/6	654
			0/6	–	0/6	–
170 °C (0.68 MPa)	0/6	–	0/5	–	6/6	480 ± 71
		–	–	–	0/6	–
		–	–	–	0/6	–

[a] Pressure at each temperature.
[b] Number of mice positive for BSE transmission per population of inoculated mice.
[c] Mice who did not have BSE until 850 d post inoculation.

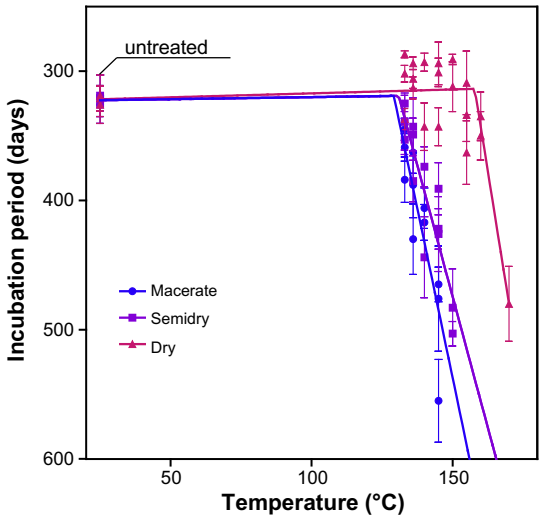

Fig. 1. Graph of incubation period and temperature for macerate, semi-dry and dried samples heated at 133 °C to 170 °C. Biphasic curves have been fitted to the data according to the formula Y1 = intercept 1 + slope 1 * X; Y at X_0 = slope1*X_0 + intercept 1; Y2 = Y at X_0 + slope2*(X - X0); Y = IF (X < X_0, Y1, Y2) using GraphPad Prism.

processing temperatures up to 155 °C could not be determined quantitatively because there were abnormally short incubation periods in the untreated and treated dry materials, as short as about 290 d post inoculation. They probably did not represent an increase in BSE titre. The data suggest that infectivity was not reduced below 150 °C but there was significant loss at and above 160 °C. At 165 °C and 170 °C infectivity fell to approximately 2.5-log LD_{50}/g.

3.3. Quantitative PMCA of heat-treated BSE tissues

PrP^{Sc} signals could be detected after PMCA in the BSE macerate treated with up to wet-heat exposure at 170 °C (Supplementary Fig. S1). The end-point titration assay of PMCA in the untreated BSE macerate showed that the positive ratio of PrP^{Sc} signals reached a plateau at the 3rd round of PMCA. PrP^{Sc} signals were not detected in the non-seed control (Fig. 2A). PrP^{Sc} signals were detected after PMCA in 2/4 samples of −8 log diluted untreated BSE macerate but not in the −9 log or the −10 log dilutions. A titer of seeding activity in the untreated BSE macerate was calculated as 10.70-log $PMCA_{50}$/g by quantitative analysis. In the examples shown BSE macerates were subjected to PMCA at different dilutions after wet-heat treatment at 133 °C, 150 °C, 155 °C, and 170 °C. PrP^{Sc} signals were detected in the −6, −5, −3, and −1 log dilutions, respectively (Fig. 2B). The seeding activity titers in 1 g BSE macerate were 8.70-log $PMCA_{50}$/g for 133 °C, 7.45-log for 150 °C, 5.70-log for 155 °C, and 3.45-log for 170 °C. Calculating each log $PMCA_{50}$/g value at every processing temperature showed that the titer decreased with increasing wet-heat temperatures (Fig. 2C).

3.4. Relationship between log reduction of infectivity and PrP^{Sc}

The limits of detection differ between the bioassay and PMCA; thus, these data could not be compared directly. The log reductions (n/n_0) of $PMCA_{50}$/g and LD_{50}/g were determined for simple comparison, where n_0 was the value of the untreated BSE macerate and n was each value after wet-heat treatment. The log reductions at 133 °C were from −2.00 log to −1.33 log of LD_{50}/g of infectivity and from −2.00 log to −1.50 log of $PMCA_{50}$/g in seeding activity. At 136 °C, 140 °C, 145 °C, and 150 °C, the log reduction values for $PMCA_{50}$/g were similar to those for LD_{50}/g (Table 3). The relationship between the log reduction in LD_{50}/g and $PMCA_{50}$/g was a

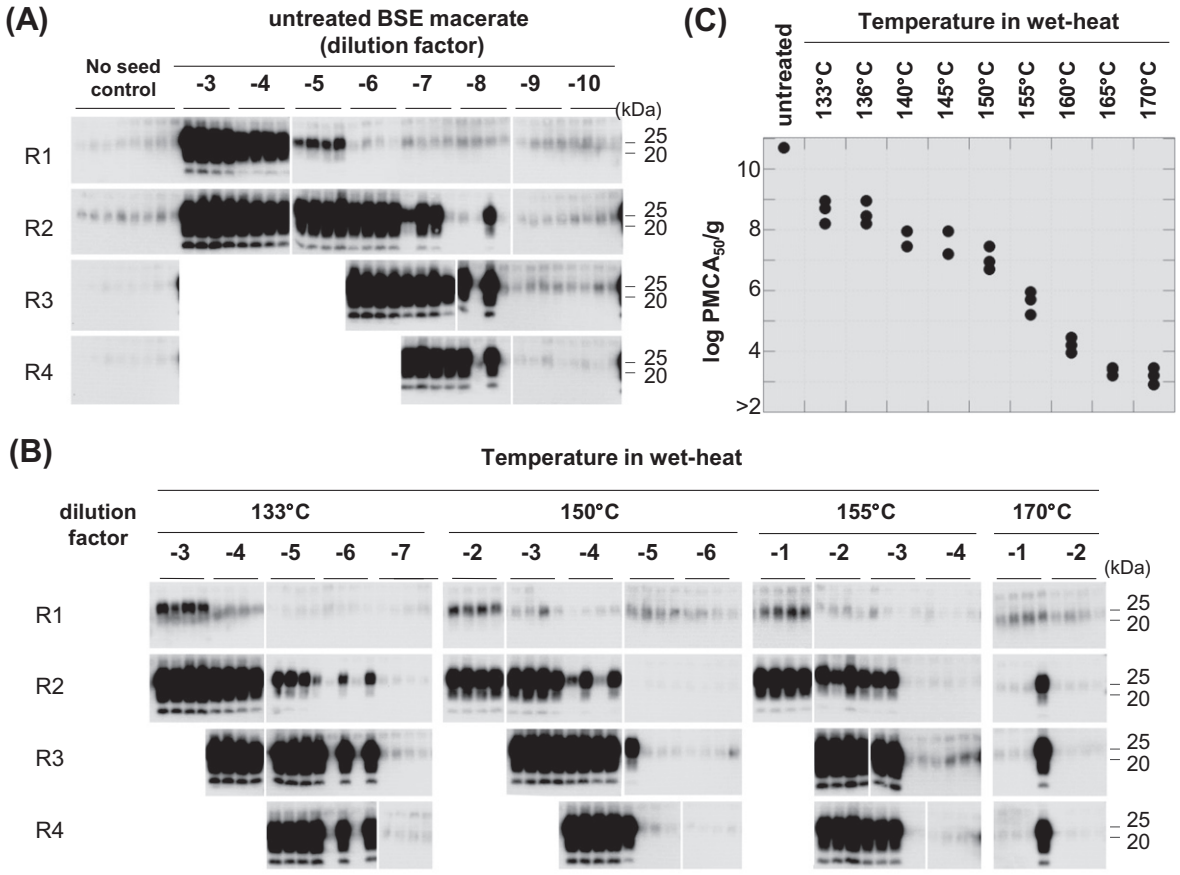

Fig. 2. PrP^{Sc} signals in PMCA with end-point titration. The PMCA assay was performed until the 4th round (R1, round 1; R2, round 2; R3, round 3; R4, round 4). PMCA products in 10-fold dilutions of the untreated (A) and treated (B) BSE macerates were examined by Western blotting after proteinase K digestion. The blots in (B) are representative samples for 133 °C, 150 °C, 155 °C, and 170 °C. The log $PMCA_{50}$/g of seeding activity titers (solid circle) were plotted for every temperature examined in triplicate (C).

strong correlation ($r = 0.86$, $p = 0.00015$) based on Pearson's correlation coefficient test. However, the relationship could not be determined at 155 °C and higher as the limit of calculable infectivity was reached at this point.

4. Discussion

These data indicate that wet-heat temperatures ⩽150 °C were not sufficient to reduce the infectivity of BSE cattle tissue macerate below the limit of detection of a highly sensitive bioassay in bovinized transgenic mice (Tables 1 and 2). The BSE infectivity was calculated in the macerate after wet-heat treatment at temperatures ranging from 133 °C to 150 °C and fell by around 2.3-log magnitude after 150 °C wet-heat treatment. The infectivity with 150 °C wet-heat treatment was lower by −3.7 log than that for the untreated samples, suggesting that BSE infectivity in the cattle tissues resists wet-heat strongly. Other studies have had similar results showing that the reduction in BSE infectivity was −2.5 log after 134 °C for 18 min [6, 8]; −2.9 log after 134 °C for 15 min and −3.2 log for 30 min [9].

We have demonstrated that wet-heat treatment at 155 °C and higher efficiently inactivates the infectivity in BSE tissue macerates to below the detection limit of this bioassay. The reduction by 155 °C wet-heat treatment was estimated as in excess of −4 log in the macerate. However, dehydration of the sample disturbed the inactivation of BSE infectivity by wet-heat treatment, as infectivity was still observed in the semidry samples at 155 °C wet-heat treatment. The infectivity in dry samples remained even at 170 °C wet-heat treatments with irregular reductions between 133 °C and 155 °C (Fig. 1). These data show that tissue dehydration may facilitate protein aggregation, which prevents the penetration of heat vapor into infectious components [16] or the removal of the water of solvation from protein components of the BSE agent [17]. It is known that TSE inactivation by autoclaving is influenced by the presence of fat, the hydration state, fixation, and dehydration of tissues, thus demonstrating the importance of hydrolysis for ameliorating TSE infectivity [5,8,16–19]. Although there are no data points below 133 °C apart from the unheated control, it is only possible to calculate the temperature at which infectivity decreases. As shown in Fig. 1, the relationship between inactivation and wet-heat temperature imply that temperature required for the inactivation of BSE infectivity increases 20 °C and higher if the cattle tissues are dehydrated. To facilitate efficient inactivation of BSE infectivity by wet-heat treatment, it is important to avoid the dehydration of cattle tissues prior to this treatment.

Quantitative PMCA analysis helped evaluate seeding activity of BSE macerates treated at temperatures up to 170 °C; these have been indicated as PrP^{Sc} signals detected on Western blotting of the PMCA products in Fig. 2. In the end-point titration assay, PrP^{Sc} signals after PMCA were detected in the −8-log dilution of the untreated BSE macerate versus the bioassay detectable in −4-log dilution, implying −4-log below the detection limit of the bioassay (Table 1). A strong correlation ($r = 0.86$, $p < 0.01$) between log reduction in LD_{50}/g and $PMCA_{50}$/g was apparent in BSE macerates treated with wet-heat from 133 °C to 150 °C (Table 3). The correlation does not necessarily reflect changes in PrP^{Sc}, which were found to diverge in previous studies [5,20]. According to the prion

Table 3
Log reductions in wet-heat treated BSE macerate.

Temperature	Log reduction [a](n/n_0)	
	in LD_{50}/g	in $PMCA_{50}$/g
(untreated macerate)	0.00	0.00
133 °C	−2.00	−2.00
	−1.33	−2.25
	−1.57	−1.50
136 °C	−1.64	−1.75
	−3.03	−2.25
	−2.09	−2.00
140 °C	−2.83	−2.75
	−2.55	−2.75
145 °C	−3.57	−3.50
	−3.19	−3.25
	−3.12	−2.50
150 °C	−3.50	−3.25
	−3.82	−3.25
155 °C	– [b]	−5.00
	–	−4.50
	–	−5.25
160 °C	–	−6.50
	–	−6.00
	–	−6.50
165 °C	–	−7.10
	–	−7.17
	–	−7.05
170 °C	–	−7.67
	–	−7.10
	–	−7.55

[A] n_0 represents the untreated BSE macerate; n represents each value after wet-heat treatment.
[b] The LD_{50}/g value was not calculated since the values fell below the bioassay detection.

hypothesis, PrP^{Sc} comprises the TSE infectious agent [1,2]. In these heat inactivation experiments the PMCA seeding properties of the infectious fraction appear to follow those of the infectious agent rather than the proteinaceous properties of PrP^{Sc}[3, 5]. In many TSE models the amount of PrP^{Sc} greatly exceeds TSE infectivity; as such, PrP, whose properties also differ from those of PrP^{Sc}, can be separated from infectivity [21–26]. If the correlation between reduction values of infectivity and seeding activity can be assumed to be below the level of detection of infectivity, reduction in infectivity of around −5.0 log magnitude at 155 °C, −6.0 log at 160 °C, −7.0 log at 165 °C, and −7.5 log at 170 °C can be predicted (Table 3). Therefore, these findings may demonstrate the potential utility of a quantitative PMCA assay for assessing the degree of BSE inactivation, since this method provides a greater dynamic range.

In conclusion, both the quantitative bioassay and PMCA have demonstrated that wet-heat treatment at 155 °C helps achieve total inactivation of BSE infectivity in intact cattle tissue. However, tissue dehydration prior to wet-heat treatment impairs efficient inactivation. Our findings provide important information on preventing BSE contamination and may also be useful for evaluating the risks of recycling cattle by-products.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.081.

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