2009

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Journal of Virological Methods 161 (2009) 168–172

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Feasibility of infectious prion digestion using mild conditions and commercial subtilisin

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Article history:
Received 7 December 2007
Received in revised form 22 January 2009
Accepted 28 April 2009
Available online 23 May 2009

Keywords:
Prion
Decontamination
Mouse-adapted scrapie
Subtilisin
Chronic wasting disease

A B S T R A C T

Two serine protease enzymes, subtilisin 309 and subtilisin 309-v, were used to digest brain homogenates containing high levels of prion infectivity using mildly alkaline conditions to investigate prion decontamination methods. To establish that PrP^{res} infectivity was eliminated, we utilized the Rocky Mountain Laboratory (RML) mouse-adapted scrapie model system for bioassay. Only one digestion condition (subtilisin 309 at 138 mAU/ml, 55 °C and 14 h digestion time pH 7.9) was considered to be highly relevant statistically ($P < 0.001$) compared to control, with 52% of challenged mice surviving until the end of the study period. In contrast, treatment of PrP^{res} by autoclaving at 134 °C or treatment with hypochlorite at a concentration of 20,000 ppm completely protected mice from prionosis. Further, in vitro assays suggest that potential proteolytic based PrP^{res} decontamination methods must use high enzyme concentration, pH values >9.0, and elevated temperatures to be a safely efficacious, thereby limiting applicability on delicate surgical instruments and use in the environment.

Published by Elsevier B.V.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are progressive diseases for which aberrantly folded prion proteins (PrP^{res}) appear to be the causative agent (Prusiner, 1982). The unique nature of PrP^{res}, the many unanswered questions regarding TSEs, and the resistance of prions to decontamination, make containment of TSEs problematic. As there are no effective treatments for prion diseases, methods to decontaminate infected surfaces and sites are especially important for managing TSEs.

A variety of methods have been tested for prion destruction (Fichet et al., 2004; McDonnell and Burke, 2003). The prion decontamination methods currently suggested in The Biosafety in Microbiological and Biomedical Laboratories manual (USDHHS, 2007), such as high concentrations of hypochlorite or 1N NaOH, are inconvenient because of noxious or irritating fumes and in many situations are not possible due to incompatibility with contaminated surfaces, materials, and equipment. Clearly, better methods for decontamination of PrP^{res} are needed.

In spite of the resistance of PrP^{res} to proteolytic inactivation, some proteolytic decontamination methods have been investigated with variable success. Protease digestion and elimination of ELISA or Western-blot immunoreactivity has been shown under a variety of conditions (Hui et al., 2004a, 2004b; Langeveld et al., 2003; Tsiroulnikov et al., 2004; Mitsuiki et al., 2006; Rapp et al., 2006; Scherbel et al., 2006; Muller-Hellwig et al., 2006). However, protease destruction of prions below in vitro assay detection limits does not necessarily mean that PrP^{res} has been reduced to levels that are no longer infectious in more sensitive bioassays; therefore, methods investigating decontamination of infectious prions need to be tested in vivo until better in vitro methods are developed and validated (McLeod et al., 2004; Lawson et al., 2006).

Bioassays in live animals are more sensitive, test actual infection, are representative of natural infection, and can detect low-level residual infectivity that may reside below the limit of detection of traditional immunoassays. Experiments utilizing mouse bioassays to investigate decontamination of PrP^{res} material with proteinase K (Jackson et al., 2005; Yakovleva et al., 2004), Properase (McLeod et al., 2004), and Rapid Multi-Enzyme cleaner 3 M (Lawson et al., 2006), suggests that enzymatic approaches to PrP^{res} contamination is achievable. Although the references cited were somewhat successful, the difficulty in achieving a robust PrP^{res} decontamination method is also revealed in the published data.

The enzymatic digestion methods presented in this paper were used as an initial screen to test for economic feasibility, mild diges-

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0166-0934/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jviromet.2009.04.040

Table 1
Conditions for treatment of mouse-adapted scrapie brain prior to injection into mice.

Group	Prion	Treatment	Concentration	Time	Temp.	Inactivation
Untreated	1:100 RML	None	NA	NA	NA	No
Negative control	Saline	None	NA	NA	NA	No
Heated control	1:100 RML	None	NA	NA	NA	Yes
Subtilisin 309 high	1:100 RML	Subtilisin 309	138 mAU/ml	14 h	55 °C	Yes
Subtilisin 309 med	1:100 RML	Subtilisin 309	70 mAU/ml	14 h	20 °C	Yes
Subtilisin 309 low	1:100 RML	Subtilisin 309	30 mAU/ml	14 h	20 °C	Yes
Subtilisin 309-v	1:100 RML	Subtilisin 309-v	138 mAU/ml	14 h	20 °C	Yes
Hypochlorite	1:100 RML	Household bleach	40% (v/v)	1 h	20 °C	No
Autoclave	1:100 RML	Autoclave	NA	20 min	134 °C	No

All treatment groups were injected with 1:100 final concentration of infectious brain homogenate the negative control group which was given phosphate buffered saline. Heat inactivation was 85 °C for 35 min.

tion buffers, and readily achievable environmental conditions in reactions to proteolytically degrade PrP^{res}. Therefore, subtilisin 309, manufactured under the commercial name Savinase (Novozymes, Bagsvaerd, Denmark), and subtilisin 309-v (Tindbaek et al., 2004), an engineered modification of subtilisin 309 to maintain high proteolytic activity at lower temperatures, were used at commercially economical enzyme concentrations, slightly basic digestion conditions and low temperatures. The conditions tested reveal that enzymatic degradation of PrP^{res} will be difficult to achieve unless high; pH, enzyme concentrations, and temperatures are used.

2. Materials and methods

2.1. Enzymes

Two enzymes: subtilisin 309 of *Bacillus clausii* (subtilisin 309), and a variant wherein valine at position 66 and serine at position 104 were substituted with alanine (subtilisin 309-v) to enhance proteolytic activity at lower temperature were used in this study to investigate enzymatic PrP^{res} decontamination. Novozymes (Denmark) provided both enzymes. All enzyme units are reported as Anson units per ml (AU/ml).

2.2. ELISA and Western blot detection of PrP^{res}

To evaluate chronic wasting disease (CWD) enzyme digestion conditions mule deer (*Odocoileus hemionus*) brain (Colorado Division of Wildlife, Fort Collins, CO) that had a predetermined PrP^{res} concentration of 3 μg PrP^{res}/gram (Raymond et al., 2000) was used. Brain material was dounce homogenized at a 1:5 (w/v) concentration in water. Digestion reactions were carried out with 6.0×10^{-5} μg PrP^{res} in 25 mM Bicarbonate buffer at the indicated pH in a total volume of 100 μl and incubated at 20 °C for 14 h. Enzyme was heat inactivated at 85 °C for 35 min. Untreated control was carried out in a parallel reaction in Delbecco's phosphate buffered saline. Evaluation of the remaining immunoreactive PrP^{res} was evaluated at the Colorado State University Veterinary Diagnostic Laboratory using the BioRad procedure and BSE test kit (BioRad, Hercules, CA) under validated standard operating procedures approved by the USDA. All samples were run in triplicate wells. CWD brain homogenate used in Western blot samples were treated as described above except no subtilisin enzyme was added. After incubation and inactivation 20 μl of samples were mixed with 5 μl 5× gel loading buffer heat denatured and resolved on 4–20% gradient SDS-PAGE gels (Invitrogen, CA). Resolved samples were transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked and with 5% (w/v) powdered milk in TBST (25 mM Tris, 140 mM NaCl, 3 mM KCl, 0.05% Tween-20, pH 8.0). Primary antibody Bar 224 was added (SpiBio, Montigny-Le-Bretonneuxx-Cedex, France) at 1/2000 dilution in TBST and 1% milk for 2 h at room temperature. Blots were washed 3× 5 min and exposed to a 1/10,000 dilution of secondary antibody (Antimouse HRP conjugated, Pierce, Rockford, IL, USA) for 1 h. Blots were washed as above and samples were visualized by chemiluminescence (Super Signal, Pierce, Rockford, IL, USA) and photographed with a CCD camera.

2.3. Digestion of material for in vivo mouse experiment

Rocky Mountain Laboratory mouse-adapted scrapie strain was used (Rocky Mountain Laboratories, Hamilton, MT) for all in vivo testing. End-stage clinical positive brain was homogenized in PBS at 1:10 (w/v) using a hand held dounce. Homogenized brain material was further diluted in PBS to a final concentration of 1:100 (w/v). Treatment conditions used for decontamination are outlined in Table 1.

2.4. Mouse inoculation and monitoring

C57Bl/6 mice (Hilltop Laboratory Animals, Hilltop, PA) were injected with 50 μl of treated or control brain homogenate in to the peritoneal cavity. The peritoneal route of challenge was chosen due to toxicity concerns of intracranial injections that could result from hypochlorite and residual enzyme matrix. Mice were monitored for kyphosis, ataxia, stiff tail, lack of grooming, emaciation, extreme lethargy/excitability; counting one point for each sign that was suspect, and two points for each distinct sign. When the condition of a mouse resulted in a score of ≥8 points, or when a mouse had ≥6 points for 3 days, the mouse was euthanized (hereafter referred to as death for simplicity). All animals were sacrificed at 18 months after challenge inoculation. All mouse experiments were conducted with the approval of the National Wildlife Research Center animal care committee and adherence to animal. welfare guidelines.

2.5. Statistical analysis

Survival analysis was performed on mouse survival data using the Log Rank test. Calculations were performed according to the British Medical Journal online survival analysis statistics instructions (eBMJ – Statistics at Square One: Survival analy-

Table 2
Survival of mice inoculated with treated mouse-adapted scrapie.

Treatment Group	Total	Died	Range	Mean ± S.D.	*P*-value
Untreated	5	5	213–213	213 ± 0	.33
Saline control	21	0	–	–	<.0001
Heated control	21	20	220–262	234 ± 11	–
Subtilisin 309 138 mAU/ml	21	9	239–261	248 ± 8	<.0001
Subtilisin 309 70 mAU/ml	18	16	224–253	233 ± 8	.58
Subtilisin 309 30 mAU/ml	21	19	223–249	233 ± 8	.71
Subtilisin 309-v 138 mAU/ml	16	13	227–255	241 ± 9	.028
Hypochlorite	21	0	–	–	<.0001
Autoclave	21	0	–	–	<.0001

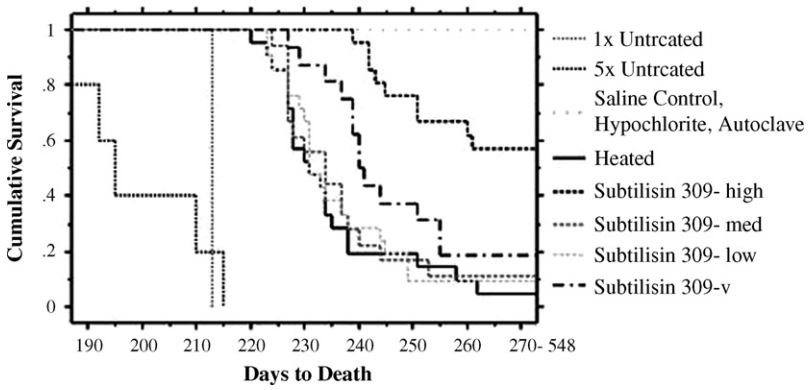

Fig. 1. Kaplan–Meyer survival curves of mice injected with treated PrP^{res}. Days 190 to 270 post inoculation, the times when mice succumbed to disease, are shown. No mice died of prion disease between 270 days and the 18-month termination of the study.

sis [http://bmj.bmjjournals.com/statsbk/12.shtml]). Excel (version 2002; Microsoft Corporation) was used to obtain *P*-values from the chi-squared values and calculate standard errors on ELISA results.

3. Results

3.1. *In vivo testing of enzymatic destruction of PrP^{res}*

The different treatment groups and the conditions for PrP^{res} digestion used in the mouse bioassay are detailed in Table 1. All mice injected with untreated RML scrapie brain homogenate were dead by 215 days after inoculation, which is typical for a high-infectivity titer RML prion strain given intraperitoneally (Pilon et al., 2007). Table 2 presents the survival statistics. Surprisingly, the heat treatment used to inactivate the enzymes prior to injection led to a 21-day increase in mean survival time relative to the untreated control. Low (30 mAU/ml) and medium (70 mAU/ml) dose subtilisin 309 treatments did not statistically improve survival over heated control; although, an additional animal survived to the study endpoint for both the low and medium does subtilisin 309 treatment groups relative to the heated control. Treatment with subtilisin 309-v led to longer survival than heating alone with an increase in the mean time to sacrifice by 7 days, whilst an increase of 14% in survivorship was observed (Fig. 1). The most effective enzyme digestion condition ($P < 0.0001$) was subtilisin 309 at 138 mAU/ml, 55 °C, and pH 7.9 with 14 h of treatment time. Treatment under these conditions reduced PrP^{res} infectivity that eliminated disease in 57% of the mice, representing a 52% increase in survivorship compared to the heated control. Two recommended methods for PrP^{res} decontamination, hypochlorite (40% bleach) treatment and autoclaving (134 °C, 20 min), eliminated all measurable PrP^{res} infectivity in this bioassay and none of the mice in these groups died of prion disease by 18 months when the study was terminated.

3.2. *Digestion of PrP^{res} (CWD) at alkaline pH*

Since subtilisin enzymes are alkaline proteases and have increased activity at higher pH, and the fact that PrP^{res} decontamination procedures are needed to treat paddocks/environments to eliminate chronic wasting disease (CWD) PrP^{res} infectivity, an in vitro ELISA assay was done to test the effect of pH and enzyme concentrations on PrP^{res} digestion using CWD infected mule deer brain homogenate. As expected, pH has a very large influence on subtilisin activity and prion degradation as shown in Fig. 2. Absorbance readings were 4-fold lower at pH 10.5 compared to pH 8.2 when the final concentration of enzyme activity was 0.6 mAU/ml for subtilisin 309. The strong pH dependence on digestion efficiency is reduced at higher enzyme concentration, with only a 2-fold and 1.5-fold reduction in ELISA absorbance observed between the high and low pH PrP^{res} digestion reactions at 6.0 and 60 mAU/ml respectively. The performance of subtilisin 309-v was less effective across all pH values as shown in Fig. 2B. A reduction of immunoreactive signal and CWD prion degradation is expected due to pH effects alone. Therefore a Western blot was run to visualize the qualitative pH dependence on PrP^{res} and is shown in Fig. 2C. The results show that pH alone reduces the immunoreactive signal by signifi-

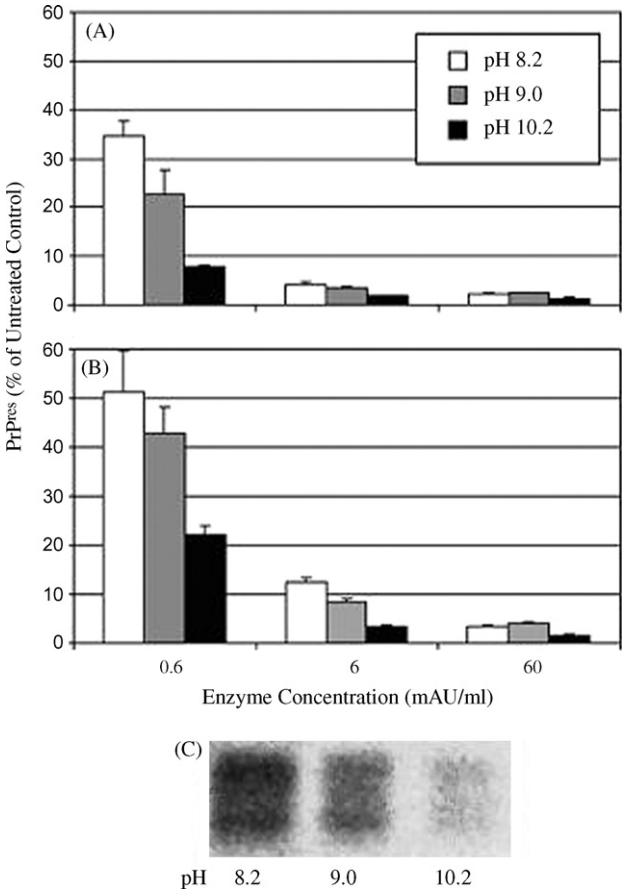

Fig. 2. (A) pH effects on subtilisin 309 and (B) subtilisin 309-vPrP^{res} degrading activity measured by BioRad BSE ELISA and (C) Western blot pH dependence on immunoreactive signal. The results are expressed as percent of untreated positive brain due to the additive effects of increased pH on immunoreactivity and PrP^{res} degradation. CWD positive mule deer brain was incubated at 20 °C for 14 h. All samples water treated at 85 °C for 35 min prior to ELISA to inactive remaining enzymes.

cant amounts. Densitometry measurements of Western blot signal showed a decrease of 30% and 60% at pH = 9.0 and 10.2 respectively relative to pH = 8.2.

4. Discussion

Our choice of bioassay conditions were largely defined by two considerations: (1) conditions had to be mild, for use on sensitive equipment or in environments where high pH may destroy microbes important for ecology; (2) enzyme concentrations needed to be held relatively low so decontamination methods could be applied economically by the end-user. To this end, even though PrP^{res} digestion conditions for the bioassay were chosen based on loss of immunoreactivity in vitro (data not shown), no significant difference was observed in survivorship between subtilisin 309 PrP^{res} digestions using 30 or 70 mAU/ml and the survivorship that was achieved by heating the homogenate to 85 °C for 35 min. Indeed, even the simple heating of PrP^{res} increased survivorship by 18 days relative to the untreated control a result that was wholly unexpected. However, the delay in end-stage clinical symptoms is not without precedence, as a similar extension in life span upon heating of PrP^{res} strains was observed by Taylor et al. (2002). The observed increase in lifespan was hypothesized to occur in the cited paper due to an alteration the structure PrP^{res} strains that results in alternative processing routes by the body compared to unheated PrP^{res} strains.

A statistically significant increase in survivorship was observed when enzyme concentration was increased and a higher digestion temperature was used. An infectious dose titration performed at Rocky Mountain Laboratories using the same prion strain and route of challenge in C57Bl/10 mice (Priola and Ward, personal communication) showed no deaths in mice inoculated with a $1{:}10^6$ dilution of infectious brain material. Our high concentration subtilisin 309 treatment showed a higher percent survival than the $1{:}10^5$ brain homogenate dilution point, indicating that our treatment was less than one log away from removal of infectivity as observed by clinical end-stage bioassay. Although it must be noted that the major histocompatibility complex difference in the congenic strains of mice may alter susceptibility to PrP^{res} challenge, it is probable that the pattern of response to decreasing doses is similar.

In addition to mouse bioassay, digestions using a CWD PrP^{res} strain with endpoint ELISA readout were conducted with pH and enzyme concentration as variables. The result of the in vitro assay showed the expected pH dependence on enzyme activity with increasing alkalinity. The observation that pH dependent dose response greatly decreases with increasing enzyme concentration suggests that a resistant core of PrP^{res} infectivity may exist below the limit of quantitation (LOQ) of the ELISA assay, as raw absorbance is still slightly above background. As others have observed, in vitro results are not predictive of *in vivo* results (Jackson et al., 2005; Kocisko et al., 1995). Therefore, to conclude if the conditions used in our in vitro digestions conditions removed all detectable CWD PrP^{res} infectivity, a bioassay using transgenic cervidized mice will be required. Of note is the fact that the observed decrease in PrP^{res} dependent ELISA signal is additive with both enzyme concentration and increasing pH playing a critical role in decontamination reactions (Fig. 3). Western blot analysis of the effect of pH on PrP^{res} quality and quantity did indicate significant alterations in PrP^{res} levels.

The results presented herein reveal that enzymatic digestion at near neutral pH, low activity, and moderate temperatures is not a viable option for PrP^{res} decontamination. If optimum alkaline pH, high enzyme concentrations and elevated temperatures are used, PrP^{res} decontamination may be possible, although the broad-based practicality from an economic and operational standpoint would likely be limited. For enzymatic degradation of PrP^{res} to be a useful tool, significant improvements are needed. For the particular enzymes used in this study, increasing pH may be a simple and effective improvement. Indeed, in vitro results show that increasing the pH of the digestion buffer as little as 1.0–1.5 pH units can dramatically improve the enzymatic digestion of PrP^{res}. In addition, additives such as detergents or chaotropic salts could also improve the enzymatic digestion of PrP^{res} and allow for viable and economical PrP^{res} decontamination method.

Acknowledgments

We thank Sue Priola and Anne Ward for providing the mouse-adapted scrapie and the survival data on the infectious material dilutions, Terry Spraker and Mike Miller for providing CWD brain material, Kathi Wilson for running the BioRad ELISA, and Steffen Ernst and Garrett Screws from Novozymes for their support and advice.

Mention of companies or commercial products does not imply recommendation or endorsement. Product names are mentioned solely to report factually on available data and to provide specific information.

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