Journal of Biotechnology & Biomaterials

Hirata et al., J Biotechnol Biomater 2015, 5:3
DOI: 10.4172/2155-952X.1000194

Research Article Open Access

Effects of Surfactant and a Hyperthermostable Protease on Infectivity of Scrapie-Infected Mouse Brain Homogenate

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Abstract

PrP^{Sc} is thought to be the infective agent of TSE, and inactivating the infectivity of PrP^{Sc} without using strong reagents is difficult. Although PrP^{Sc} is a protease resistant protein, it can be degraded *in vitro* by the hyperthermophilic protease (Tk-subtilisin) at temperatures above 65°C through the synergistic effect of heat destabilization of PrP and the high proteolytic activity of the thermostable protease. However, the change in infectivity of the protease-digested PrP^{Sc} is still unknown. Therefore, we used mouse brain homogenate containing PrP^{Sc} (SBH) in a bioassay to investigate the loss of infectivity after Tk-subtilisin digestion. Surprisingly, the Tk-subtilisin digested SBH retained a high level of infectivity. Despite this, Tk-subtilisin could still be used for decontamination in highly protein denaturing condition such as in the presence of SDS.

Keywords: Protease; Thermostable enzyme; Prion; Scrapie; Tk-subtilisin

Abbreviations: PrP: Prion Protein; PrP^{Sc}: Scrapie-Associated PrP; PrP^{C}: Cellular PrP; TSE: Transmissible Spongiform Encephalopathy; CJD: Creutzfeldt - Jakob disease; SBH: Mouse Scrapie (Strain Chandler) Brain Homogenate; PK: Proteinase K; SDS: Sodium Dodecyl Sulfate; GdnHCl: Guanidine Hydrochloride; DTT: Dithiothreitol; SDW: Sterilized Distilled Water

Introduction

An abnormal prion protein with a β-sheet-rich conformation, designated scrapie-associated prion protein (PrP^{Sc}) [1], is the major protein component of an infective prion associated with transmissible spongiform encephalopathies (TSEs) [2]. PrP is distinguished to PrP^{C} and PrP^{Sc} by its infectivity and they have different physical properties such as Proteinase K sensitivity and solubility. PrP^{Sc} is partially resistant to Proteinase K digestion and favors to form various oligomers [1,3]. PrP^{Sc} is formed from PrP^{C} through its structural change from an α-rich conformation to a β-rich conformation [4]. PrP^{Sc} induces the structural change of PrP^{C} by binding and being a template of new PrP^{Sc} molecule. Thus PrP^{Sc} is a proteinaceous, self-propagating molecule [5-7].

Since PrP^{Sc} is resistant to heat denaturation at 121°C and many chemical decontamination methods, PrP^{Sc} inactivation is an important research target with the objective of TSE prevention. The World Health Organization recommends a combination of cleaning, chemical treatment, and heat sterilization of medical equipment [8]. More specifically, the guidelines recommend the use of an autoclave and strong chemical treatments, such as high concentration sodium hydroxide or sodium hypochlorite, for reusable instruments. Although these procedures are effective for eliminating infectivity, some surgical and complex instruments such as fiber optic endoscopes cannot be decontaminated using these methods because they could be damaged [9]. Furthermore, the safety risks associated with the use of strong chemicals are of concern to the medical community [10]. Therefore, prion decontamination procedures with sufficient potency and improved safety are required [11].

Subtilisin family proteases from *Bacillus* sp. are often used in cleaning reagents for medical equipment as an active element for removing contaminating protein. Okoroma et al. found that keratinase from *Bacillus licheniformis* can degrade PrP^{Sc} [12], and other subtilisin variants are in practical use as prion decontamination agents that can be used in moderate conditions such as at 50°C. In addition, hyperthermostable proteases have been tested for PrP^{Sc} degradation. Snaider et al. used an extracellular extract of *Aeropyrum pernix* for *in vitro* digestion of PrP^{Sc} in CJD infected brain homogenate [13]. Takano and Koga found that two subtilisin family proteases from *Thermococcus kodakarensis* KOD1, Tk-subtilisin and Tk-SP, degrade PrP^{Sc} in mouse scrapie (Chandler strain)-infected brain homogenate (SBH) to a level undetectable by western blot analysis [14]. Tk-subtilisin is a subtilisin-like serine protease which has significant heat stability, with its highest specific activity at 90°C [15,16]. Tk-subtilisin can digest proteins under severe physical conditions in which most proteins are denatured. Since PrP^{Sc} is thought to be destabilized at high temperature, hyperthermophilic proteases have promising potential as active ingredients of medical detergents for prion decontamination. However, there is no report evaluating the loss of infectivity of protease digested PrP^{Sc}. In this study, we used a bioassay to evaluate the infectivity of SBH treated with Tk-subtilisin under various conditions.

Materials and Methods

Preparation of proteases

Proteinase K (PK) was purchased from Wako Pure Chemicals Ltd, Osaka, Japan. Tk-subtilisin was prepared from recombinant *E. coli* BL21 (DE3) harboring the Tk-subtilisin gene expression vector, as described previously [14,17].

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Received July 22, 2015; **Accepted** August 25, 2015; **Published** August 31, 2015

Citation: Hirata A, Sakudo A, Takano K, Kanaya S, Koga Y (2015) Effects of Surfactant and a Hyperthermostable Protease on Infectivity of Scrapie-Infected Mouse Brain Homogenate. J Biotechnol Biomater 5: 194. doi:10.4172/2155-952X.1000194

Preparation of mouse brain homogenate and western blotting

Brain homogenate of terminally-diseased mice infected with the Chandler strain of scrapie prion (SBH) was prepared at 10% (w/v) in sterile PBS. The protein concentration of the homogenate was measured using a DC protein assay kit (BioRad). To prepare the sample for PrP degradation, an appropriate amount of the homogenate, equivalent to 60 µg of protein, was mixed with 0.5 M Tris-HCl (pH 8.0) and the required concentrations of Tk-subtilisin and distilled water, such that the total volume was 50 µL for each condition. Sodium dodecyl sulfate (SDS) at 3% (w/v) was added as necessary. The resultant samples were incubated at 100°C for the specified time. When inactivation of Tk-subtilisin was required, 50 mM diisopropylfluorophosphate was added prior to sample preparation for SDS polyacrylamide gel electrophoresis (SDS-PAGE). Two-times loading buffer (150 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% (w/v) glycerol, and 0.03% (w/v) bromophenol blue) was added and the samples were boiled for 5 min. SDS-PAGE was performed using a 15% polyacrylamide gel, and PrP was detected by western blotting with an anti-PrP antibody, SAF83 (SPI bio, Montigny le Bretonneux, France).

Bioassay of infectivity

All animal studies were carried out in accordance with the guidelines for animal experiments of the School of Health Science, Faculty of Medicine, University of the Ryukyus.

A 10% w/v suspension of SBH from Chandler strain-infected mice was used to test the effect of Tk-subtilisin degradation or SDS treatment on the infectivity of PrP^{Sc}. The 10% SBH suspension was diluted to 1% with 200 mM Tris-HCl buffer (pH 8.0). Either 2.0 µg/mL of Tk-subtilisin or 1% SDS, or both, was added to each aliquot of the SBH. SBH without Tk-subtilisin and SDS was prepared as a control. Each aliquot was incubated at 100°C for 60 min to inactivate PrP^{Sc}. Each resultant aliquot was intracerebrally inoculated into 11-week-old male C57BL6/JJmsSlc mice. A total of 20 µl of 1% SBH suspension was injected into the cerebral ventricular system of mice using a micro syringe. Six mice from each inoculation group were examined for the indicated period.

Immunohistochemistry

Two mice from each inoculation group were euthanized with ether after 155 days of incubation. Brains and spleens were dissected and immersed in 4% paraformaldehyde in phosphate buffered saline for 2 days at 4°C. For light microscopy, specimens were dehydrated in a graded ethanol series and embedded in paraffin. The specimens were sliced into sections and immobilized on glass slides. The sections were deparaffinized using xylene and washed with a series of ethanol and distilled water. For histological observation, some sections were stained with hematoxylin and eosin. For immunohistochemistry, serial sections were prepared by autoclaving at 121°C for 15 min, immersing in 1 mM HCl, and then washing with 3% hydrogen peroxide solution. The sections were incubated overnight at 4°C with SAF83 (SPI bio) anti-mouse PrP monoclonal antibody diluted to 1:1000. After washing, the sections on the slides were incubated with a secondary antibody (Dako EnVision; Dako Japan Co., Tokyo, Japan) at room temperature for 30 min. Immunoreactivity was visualized by immersion in a DAB-H_2O_2 solution (Nichirei Biosciences, Tokyo, Japan). Sections were then counterstained with hematoxylin and observed under an All-in-one Type Fluorescence Microscope (BZ-8000; Keyence, Osaka, Japan) using BZ Analyzer Software (Keyence).

Results

As shown in our previous study [14], PrP^{Sc} in brain homogenate from scrapie-infected mice was degraded by a hyperthermophilic protease, Tk-subtilisin, to levels undetectable by western blot. The results show that 2.0 µg/mL of Tk-subtilisin can degrade the PrP to a level undetectable by western blotting. However, it was not known whether the degraded PrP^{Sc} had lost its infectivity. To clarify this, the infectivity of SBH treated with Tk-subtilisin under various conditions was evaluated using a bioassay. In total, six groups of mice were subjected to different inoculants, as shown in Table 1. The clinical symptoms and weight loss for each mouse from each group were observed until they died, or until 453 days post-inoculation.

Two mice from each inoculation group were euthanized at 155 day post-inoculation to compare PrP^{Sc} accumulation in their tissues. Tissue specimens were prepared from brains and spleens of each mouse, and the pathological changes in the brains and accumulation of PrP^{Sc} in the spleens was observed. Brain and spleen tissue from a non-infected and infected mouse are shown in Figure 1. The number and extent of vacuoles observed in the brain of the infected animal indicated that it was at the terminal stage of TSE disease at 155 day post-inoculation while the spleen tissues from both infected and non-infected mouse shows slight difference. The brains and spleens of mice from each test group were also observed at 155 day post-inoculation, as shown in Figure 2. To identify the mouse at the terminal stage of Scrapie, the brain tissue of each test group has been observed whether it has vacuoles

Inoculum	N/N_0 [a]	Survival rate (%)	Mean time of survival (days)
Sterilized distilled water	0/6	100	>453
1% SBH without heat treatment	4/4	0	168.5
1% SBH*	6/6	0	255.7
1% SBH* + 2 µg/mL Tk-subtilisin	6/6	0	262.7
1% SBH* + 1 % SDS	1/6	83.3	>420
1% SBH* + 2 µg/mL Tk-subtilisin + 1% SDS	0/6	100	>453

[a]N/N_0, number of mice that developed infection per number of inoculated mice.
*Samples were heat treated at 100°C for 1 hr.

Table 1: Survival times for mice infected with scrapie (Chandler strain).

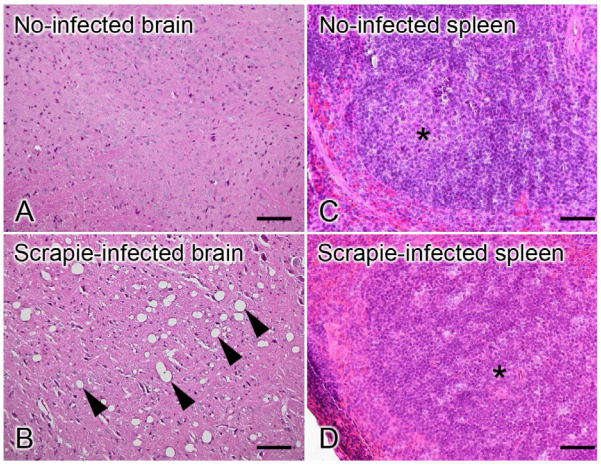

Figure 1: Light micrograph images of mouse brains (A, B) and spleens (C, D) 155 days post-inoculation stained with hematoxylin and eosin. (A, B) Many vacuoles are observed throughout all areas of the brain of a scrapie-infected mouse (arrowheads), while no vacuole is seen in the brain of a no-infected mouse. (C, D) Lymph nodules containing germinal center (asterisk) are observed in the spleens of both a no-infected mouse and a scrapie-infected mouse. The germinal center of a scrapie-infected mouse is diffusely large, suggesting an immune response to the infection. Bars=100 µm.

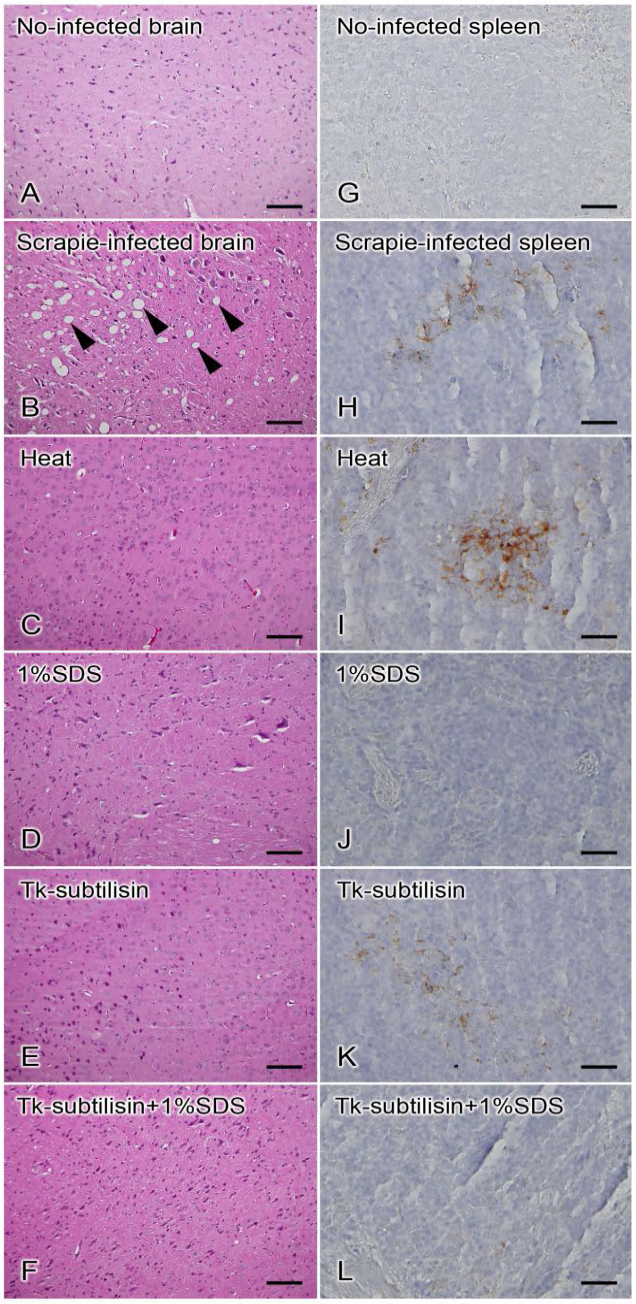

Figure 2: Light micrograph images of mouse brains (A-F) and spleens (G-L) 155 days post-inoculation. (A-F) Neuropil vacuolation is observed throughout all areas of the brain of (B) a scrapie-infected mouse (arrowheads), whereas no vacuolation is seen in the brain of (A) a no-infected mouse, (C) a mouse inoculated with SBH incubated at 100°C, (D) a mouse inoculated with SBH incubated at 100°C with 1% SDS, (E) a mouse inoculated with SBH digested with Tk-subtilisin, and (F) a mouse inoculated with SBH incubated with Tk-subtilisin and 1% SDS. (G-L) Light micrograph images showing the localization of PrP in the spleen of (G) a no-infected mouse, (H) a scrapie-infected mouse, (I) a mouse inoculated with SBH incubated at 100ºC, (J) a mouse inoculated with SBH incubated at 100°C with 1% SDS, (K) a mouse inoculated with SBH digested with Tk-subtilisin, and (L) a mouse inoculated with SBH incubated with Tk-subtilisin and 1% SDS. Immunoreactivity for PrP (brown signal) is detected around the center of the lymph nodule in the spleen of (H) a scrapie-infected mouse, (I) a mouse inoculated with SBH incubated at 100ºC and (K) a mouse inoculated with SBH digested with Tk-subtilisin. No labeling for PrP is observed in the lymph nodules (G, J, L). Bars: A-F=100 µm; G-L=50 µm.

or not. Brain tissues of each mouse inoculated SBH treated with heat, 1% SDS, Tk-subtilisin and both 1% SDS and Tk-subtilisin respectively (Figure 2C-2F), did not show vacuoles as shown in Figure 2B. However, the immunohistochemistry results showed PrP^{Sc} accumulation in the spleens of mice that were inoculated with heat-treated SBH or Tk-subtilisin-treated SBH (Figure 2I and 2K) while the other brain tissue has no PrP^{Sc} accumulation (Figure 2J and 2L). Since the spleen is the organ that accumulates the PrP^{Sc} earlier than brain, infection of scrapie can detect before the final stage of the disease.

The number of mice that survived until the end of the experiment, the survival rate, and the mean time of survival for each group are summarized in Table 1. All four mice injected with the untreated 1% SBH (positive control) lost weight from 144–155 days post-inoculation, and abnormal behaviors such as tremors and ataxia were observed in this group. The incubation period matched that of a typical scrapie-infected mouse. Because the described symptoms indicated that the mice were at the terminal stage of TSE, the mice were euthanized at 155 or 182 days post-injection (mean incubation time of 168.5 days). The mice from the groups injected with heat-treated SBH developed infections from 239–265 days post-inoculation (mean time 255.7 days), indicating that 1 h of heat treatment at 100ºC decreased the infectivity of SBH. The mice injected with Tk-subtilisin-treated SBH developed infections from 253–280 days post-inoculation (mean time 262.7 day), indicating that enzymatic digestion did not improve the heat treatment. 17 mice in the other groups survived until the end of the experimental period, indicating that the inoculant used in these groups had lost significant infectivity. However, there was one exception: a mouse from the group injected with SBH treated with 1% SDS developed TSE at 255 days post-inoculation, indicating that this treatment method did not completely eliminate infectivity.

Discussion

Based on the results, we conclude that Tk-subtilisin-treated SBH maintains its infectivity, despite the fact that western blot analysis did not show any PrP signal. It is possible that the Tk-subtilisin degraded the epitope region of the PrP^{Sc}, but that the undigested parts of PrP^{Sc} remained as infectious as the native protein. However, a similar result was observed when another anti-PrP antibody, SAF32, was used for the western blot analysis (data not shown). The epitope of SAF83 is amino acid residues 142–160 of PrP, while that of SAF32 is residues 51–91. The other possibility is that the degradation of PrP^{Sc} is quantitatively insufficient to eliminate infectivity even though it cannot be detected by western blot analysis. Further experiments are required to investigate the potential reasons for PrP^{Sc} infectivity after Tk-subtilisin digestion.

Additionally, it is likely that SDS plays an important role in the degradation of PrP^{Sc} by Tk-subtilisin. SBH includes many biogenic substances, such as nucleic acids and fatty acids, which are thought to interact with PrP^{Sc} [18], and are likely to interfere with the interaction between Tk-subtilisin and PrP^{Sc}. Furthermore, PrP^{Sc} is known to form oligomers and insoluble particles. These features may hinder proteolysis of PrP^{Sc} in SBH. Because SDS is presumed to function by enabling Tk-subtilisin to access the insoluble PrP^{Sc} particles, it is possible that the presence of SDS allows Tk-subtilisin to degrade the infective core of PrP^{Sc}, which cannot be achieved by Tk-subtilisin alone.

From the bioassay results (Table 1), it is clear that SDS treatment significantly decreases the infectivity of SBH, likely by denaturing PrP^{Sc} in SBH. However, as shown by the results of the survival test, the infectivity loss is not complete. However, treating SBH with both Tk-subtilisin and SDS decreases SBH infectivity more effectively than

SDS alone as a result of the cooperative effect of denaturation by SDS and degradation by Tk-subtilisin. Thus, Tk-subtilisin may be a useful ingredient to include in reagents designed for the decontamination of PrP^{Sc}. To probe this apparent cooperation, further quantitative analysis of Tk-subtilisin degradation of PrP^{Sc} is necessary.

Effective decontamination procedures for PrP^{Sc} in the medical field are of great importance. The use of a protease as an ingredient in medical detergents is a very attractive option for establishing a simple prion decontamination procedure. Further quantitative evaluation of the effects of Tk-subtilisin on PrP^{Sc} infectivity in less stringent conditions is imperative.

Acknowledgments

The authors wish to acknowledge Dr. Kazuyoshi Ikuta, Professor of The Research Foundation for Microbial Disease of Osaka University, for his help in interpreting the significance of the results of this study. This work was supported in part by a Grant from the Senri Life Science Foundation, Japan, by the Ministry of Health, Labour and Welfare, Japan, and by an Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan. The authors have no conflicts of interest to declare.

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