Degradation and destabilization of abnormal prion protein using alkaline detergents and proteases

YOSHIHIKO HIRATA[1*], HITOSHI ITO[1*], TARO FURUTA[1], KAZUYOSHI IKUTA[2] and AKIKAZU SAKUDO[2,3]

[1]Biochemical Laboratory, Saraya Co., Ltd., 24-12, Tamate-cho, Kashiwara, Osaka 582-0028; [2]Department of Virology, Center for Infectious Disease Control, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871; [3]Laboratory of Biometabolic Chemistry, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

Received July 22, 2009; Accepted September 29, 2009

DOI: 10.3892/ijmm_00000340

Abstract. There is a limited number of reports regarding detergents and proteases inactivating, degrading, or destabilizing abnormal prion protein (PrP^{Sc}). In the present study, the effect of alkaline detergents and proteases on the breakdown of PrP^{Sc} in the absence of proteinase K (PK) (degradation) and the presence of PK (destabilization) was investigated. PrP^{Sc} from brain homogenate of terminally-diseased mice infected with the Chandler strain of scrapie was used as a substrate. A surfactant-free alkaline detergent (pH 11.9, 1% aqueous solution) with potassium hydroxide as the main ingredient and an alkaline detergent (pH 11.9, 1% aqueous solution) containing about 1% surfactant as well as two commercially available alkaline proteases had a destabilizing effect on PrP^{Sc}. All these detergents and proteases showed degradative effects on PrP^{Sc} under appropriate conditions. These results demonstrate the usefulness of alkaline detergents and proteases for the degradation or destabilization of PrP^{Sc}.

Introduction

Prion is an infectious pathogen causing prion diseases such as bovine spongiform encephalopathy in cattle, Creutzfeldt-Jakob Disease (CJD) in humans, and scrapie in sheep (1). Prion is mainly composed of abnormal prion protein (PrP^{Sc}) that is converted from cellular prion protein (PrP^{C}) (2). As PrP^{C} is highly expressed in neurons (3,4), the amyloids are easily formed in neuron and thus, neuronal cell loss, the accumulation of PrP^{Sc}, and astrogliosis are all hallmarks of prion diseases (5). CJD, as well as some inherited prion diseases in Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia, is transmissible among humans (2). Therefore, the inactivation of prion on surgical tools and medical instruments which are re-used is an important concern for public health.

There are four known types of CJD, namely: sporadic CJD, familial CJD, iatrogenic CJD and variant CJD (6). About 90% of CJD cases are classified as sporadic, with no family history and no known source of transmission, and this form has an incidence rate of 1/million. The second most common form is familial CJD which is inherited (less than 10%). Less than 1% of CJD cases are thought to be iatrogenic or variant in nature (7). The ratio of iatrogenic CJD, which occurs through infection as a result of medical activities, is relatively small but has become somewhat of a problem in healthcare settings (8). The reason for this is that most of the sterilization processes that have been used up to now including autoclaving for 15 min at 121˚C, dry heat sterilization for 2 h at 180˚C, and treatment with glutaraldehyde and peracetic acid cannot inactivate PrP^{Sc} and thus, there is a risk of infection through medical and dental instruments (9). Up to now, it has been reported that more than 350 people have been infected through the implantation of cornea transplants, growth hormone and blood transfusions, and electrodes that come into direct contact with the brain (10,11).

The World Health Organization recommends methods such as autoclaving for 18 min at 134˚C, and immersion or boiling in strong denaturants or alkali as countermeasures to prevent CJD infection (12). However, these methods may damage or destroy medical instruments, especially fragile and expensive items such as gastroscopes for which mild sterilization is preferable (13) and thus, a PrP^{Sc} decontamination method that is effective, can be routinely applied in healthcare settings, and is compatible with that required by humans and the environment (14).

To assess the effect of treatment on PrP^{Sc}, Lemmer *et al* defined three categories; 'degradation', 'detachment' and

Correspondence to: Dr Akikazu Sakudo, Laboratory of Biometabolic Chemistry, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan
E-mail: sakudo@med.u-ryukyu.ac.jp

*Contributed equally

Key words: PrP^{Sc}, degradation, destabilization, alkaline detergent, alkaline protease

Table I. Alkaline detergents and enzyme reagents.

Product name	Abbreviation	Classification	Manufacturer
Yogore Toreiru R	YT	Alkaline detergent	Saraya Co., Ltd.
Power Quick W	PQ	Alkaline detergent	Saraya Co., Ltd.
Savinase Ultra 16XL	Savinase	Alkaline protease	Novozymes Japan Co., Ltd.
Esperase 8.0L	Esperase	Alkaline protease	Novozymes Japan Co., Ltd.

Table II. Products with an inactivating or destabilizing effect on PrP^{Sc}.

Product name	Abbreviation	Classification	Manufacturer	Recommended conditions for PrP^{Sc} (concentration/temperature/time)
Protex PRP	Protex	Alkaline protease	Genencor Co., Ltd.	2%, 60˚C, 30 min
deconex 28 Alkaone	Alkaone	Alkaline detergent	Borer Chemie AG Co., Ltd.	0.3-0.7%[a], 70-90˚C
Hamo™ 100 PID	Hamo 100	Alkaline detergent	Steris Co., Ltd.	1.6%, 43˚C, 15 min

[a]The concentration of detergent used was determined based on the hardness of water used in the healthcare facility (water quality: soft (<125 ppm), 0.3%; medium hard (125-375 ppm), 0.5%; hard (>375 ppm; 0.7%). In this study, water hardness was 50 ppm and thus, the detergent was used at a concentration of 0.3%.

'destabilization' (15). Degradation indicates that after treating PrP^{Sc} with a reagent, even without proteinase K (PK) digestion, the signal from a Western blot analysis can either only be detected in a significantly reduced amount or not be detected at all. On the other hand, destabilization indicates that the signal can be detected without PK digestion, but can either only be detected in a significantly reduced amount or not be detected at all upon PK digestion. In this report, we investigated the degradative and destabilizing effects of alkaline detergents and proteases shown in Table I on PrP^{Sc}.

Materials and methods

Detergents and proteases. Yogore Toreiru R (YT; Saraya Co., Ltd., Osaka, Japan) and Power Quick W (PQ; Saraya Co., Ltd.) were used as the alkaline detergents. The former contains 1% surfactants, has 5.6% free alkali and is often used as a detergent for floor maintenance purposes, while the latter has 11.4% free alkali and is used as a detergent dedicated for washer disinfectors (automated reprocessing machines). With respect to PQ, because it is a surfactant-free formulation, an oligolipid type of biosurfactant called sophorolipid (SL; Saraya Co., Ltd.) was added to PQ at concentrations of 0.3% (w/w) and 1% (w/w) and utilized as additional test samples (namely: PQ+0.3% SL and PQ+1% SL). Savinase Ultra 16XL (Savinase; Novozymes Japan Co., Ltd., Chiba, Japan) and Esperase 8.0L (Esperase; Novozymes Japan Co., Ltd.) were used as the protease for testing. For comparison, a product proven to be effective in inactivating PrP^{Sc}, namely: Hamo™ 100 Prion Inactivating Detergent (Hamo 100; Steris Co., Ltd., Ohio, USA), and a product proven to have a destabilizing effect on PrP^{Sc}, namely: 28 deconex Alkaone (Alkaone; Borer Chemie AG Co., Ltd., Zuchwil, Switzerland), were used. Products used in this study are summarized in Tables I and II. Protex PRP (Protex) was kindly provided by Genencor International (Palo Alto, CA, USA). With respect to all the enzyme reagents tested, test conditions were adjusted to a pH value of 12.0 using sodium hydroxide.

Sample preparation and Western blotting. Brain homogenate of terminally-diseased mice infected with the Chandler strain of scrapie was used to confirm the efficacy against PrP^{Sc}. Brain homogenate (5μl, equivalent to 60 μg of protein) in phosphate-buffered saline (PBS) was added to enough distilled water to adjust the concentration of the test sample to the designated value, such that the total volume was 50 μl. After the destabilization process was performed at the designated test concentration and temperature, the solution was transferred to an ice bath and 22 μl of 1 M phosphate buffer were added. If PK treatment was to be performed, 8 μl of 20 mg/ml PK were added for 1 h at 37˚C. If PK treatment was not to be performed, then 8 μl of PBS buffer instead of PK were added. After 80 μl of 2x loading buffer [150 mM Tris pH 6.8, 6% sodium dodecyl sulfate (SDS), 30% glycerol, 0.03% bromophenol blue] was added and heating (boiling) for 5 min at 100˚C, PrP^{Sc} was detected using SDS-polyacrylamide gel electrophoresis (PAGE) (16) and Western blotting with the anti-PrP antibody SAF83 (SPI bio, Montigny le Bretonneux, France) which recognizes residues 126-164 of PrP (17).

Results and discussion

First, we examined the effect of alkaline detergents and proteases on the degradation and destabilization of PrP^{Sc} under conditions recommended by the manufacturers (Fig. 1A): 2%, 60˚C, 30 min, pH 12.0 in Protex, 0.3%, 70˚C, 30 min, pH 11.4 in Alkaone, and 1.6%, 43˚C, 15 min in Hamo 100. A

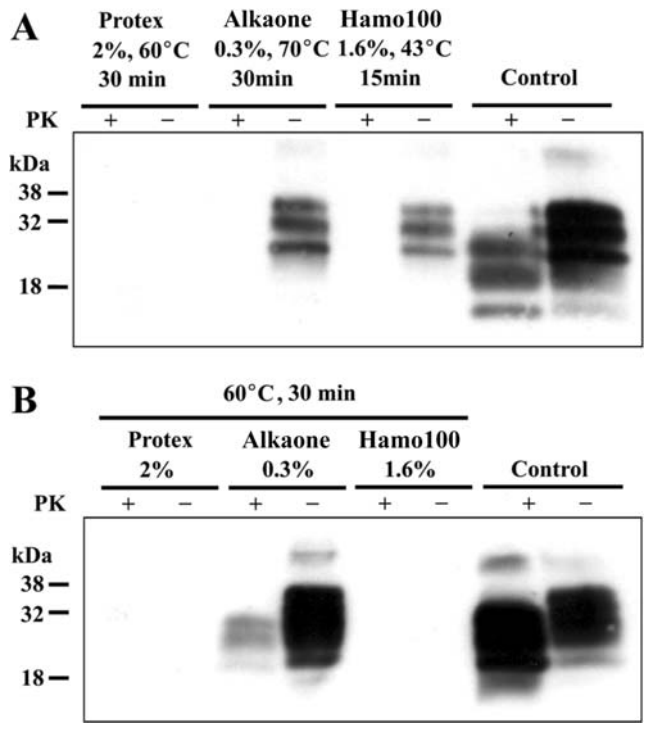

Figure 1. Degradative and destabilizing effects of alkaline protease and detergents on abnormal prion protein (PrP^{Sc}) under conditions recommended by the manufacturer or with the same incubation period. Scrapie-infected brain homogenate (chandler) was incubated in (A) 2%(w/v) Protex PRP for 30 min at 60˚C, 0.3% (v/v) deconex28 Alkaone for 30 min at 70˚C, and 1.6% (v/v) Hamo™ 100 PID for 15 min at 43˚C as well as (B) 2% (w/v) Protex 0.3% (v/v) Alkaone and 1.6% (v/v) Hamo 100 for 30 min at 60˚C. Untreated samples (control) were also included. After the incubation, proteins were incubated in the absence (-) and presence (+) of proteinase K (PK) at neutral pH (pH 7.0) for 1 h at 37˚C. The samples were further subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting with anti-prion protein (PrP) antibody, SAF83, for PrP^{Sc} or total PrP, respectively. A broad PrP^{Sc} signal with an approximate molecular weight of 15-32 kDa was observed only in the control and Alkaone-treated samples at 0.3%, 60˚C and 30 min. The levels of total PrP showing broad signals with an approximate molecular weight of 20-37 kDa were decreased with all reagents except Alkaone at 0.3%, 60˚C and 30 min. Both total PrP and PrP^{Sc} were under the detectable limit in Protex (2%, 60˚C and 30 min), Hamo 100 (1.6%, 60˚C and 30 min) treated samples. Protein molecular mass markers (kDa) are shown on the left.

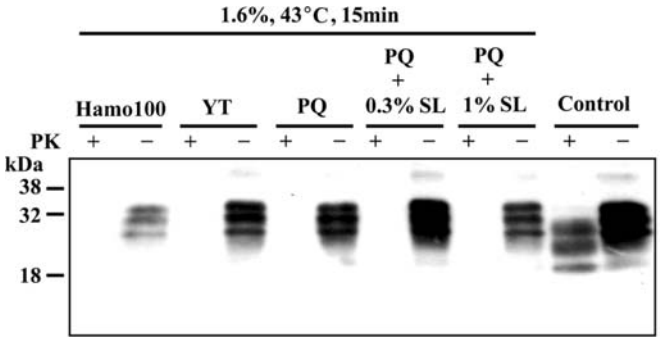

Figure 2. Comparison of degradative or destabilizing effects of alkaline detergents on PrP^{Sc} at the same concentration under the same conditions. Scrapie-infected brain homogenate (chandler) was incubated in 1.6% (v/v) Hamo 100, YT, PQ, PQ + 0.3% sophorolipid (SL; Saraya Co., Ltd.), or PQ + 1% SL for 15 min at 43˚C. Un-treated samples (control) were also included. After the incubation, proteins were incubated in the absence (-) and presence (+) of PK at neutral pH (pH 7.0) for 1 h at 37˚C. The samples were further subjected to SDS-PAGE and Western blotting with anti-PrP antibody, SAF83, for PrP^{Sc} or total PrP, respectively. A broad PrP^{Sc} signal with an approximate molecular weight of 15-32 kDa was observed in the control sample but not in detergent-treated samples. The levels of total PrP showing broad signals with an approximate molecular weight of 20-37 kDa were decreased in all detergent-treated samples. Protein molecular mass markers (kDa) are shown on the left.

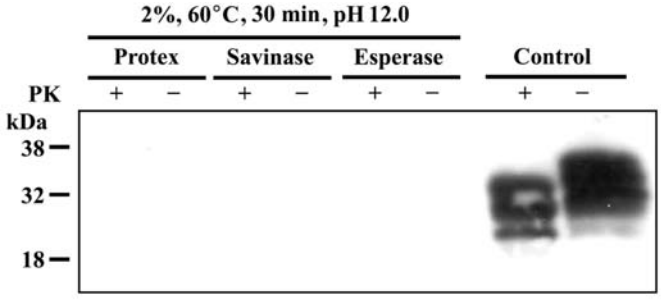

Figure 3. Comparison of degradative or destabilizing effects of alkaline proteases on PrP^{Sc} at the same concentration under same conditions. Scrapie-infected brain homogenate (chandler) was incubated in 2% (w/v) Protex, Savinase and Esperase for 30 min at 60˚C and pH 12.0. Un-treated samples (control) were also included. After the incubation, proteins were incubated in the absence (-) and presence (+) of PK at neutral pH (pH 7.0) for 1 h at 37˚C. The samples were further subjected to SDS-PAGE and Western blotting with anti-PrP antibody, SAF83, for PrP^{Sc} or total PrP, respectively. A broad PrP^{Sc} signal with an approximate molecular weight of 15-32 kDa was observed in the control sample but not in other protease-treated samples. Total PrP showing broad signals with an approximate molecular weight of 20-37 kDa, which was observed in the control sample, was also under the detectable limit in all protease-treated samples. Protein molecular mass markers (kDa) are shown on the left.

comparison suggested Hamo 100 to be more effective against PrP^{Sc} than Alkaone. Protex showed no bands of PrP in the absence or presence of PK on Western blotting of SAF83, suggesting a degradative effect of Protex. On the other hand, Alkaone and Hamo 100 indicated only destabilization because signals were obtained in the absence but not presence of PK.

Second, the detergents and proteases were subjected to an analysis of the degradative and destabilizing effect on PrP^{Sc} under the same conditions (60˚C, 30 min) (Fig. 1B). The result showed that Protex and Hamo 100 had complete degradative and destabilizing effects, whereas Alkaone had a significant but not complete destabilizing effect and no degradative effect. These results suggest that the incubation conditions are essential for sufficient degradation and destabilization.

Next, the effect of other alkaline detergents on PrP^{Sc} was investigated. YT and PQ had a fully destabilizing effect on PrP^{Sc} similar to Hamo 100 at 1.6%, 43˚C, and 15 min (Fig. 2). On the other hand, YT and PQ were no less effective than Hamo 100 in terms of the degradation of PrP^{Sc}. Regarding this discrepancy between Hamo 100 and the two detergents (YT and PQ), a high concentration of free alkali in the presence of detergent may contribute to the effectiveness of Hamo 100, because the combination of alkali and detergent is effective against PrP^{Sc}. However, further study will be needed to fully explain the discrepancy. In this study, we also

examined the combined effects of SL and PQ against PrP^{Sc}. The result demonstrated that SL did not enhance the degradation of PrP^{Sc}.

Finally, the effect of commercial alkali proteases (Savinase and Esperase) on PrP^{Sc} at 2%, 60˚C, pH 12.0, and 30 min was investigated (Fig. 3). Interestingly, both Savinase and Esperase degraded and destabilized PrP^{Sc} under the conditions studied.

Several alkaline detergents and enzyme reagents have proven effective in inactivating or destabilizing PrP^{Sc}, and are currently available on the market. The subtilisin-enzyme Properase is reported to be able to degrade PrP^{Sc} at high temperatures under alkaline conditions (18), while a keratinase from *Bacillus licheniformis* requires >100˚C and the presence of a detergent to break down PrP^{Sc} (19). An alkaline protease, E77, derived from *Streptmyces* species has shown degradation of PrP^{Sc} at 60˚C and pH 11.0 (20). Hamo 100 and Properase were proved to eliminate prion infectivity (18,21). An alkaline detergent, high pH and high temperature may destabilize the rigid structure of PrP^{Sc}, and are expected not only to remove PrP^{Sc} from medical instruments, but also to prevent its reattachment (21).

Moreover, although some studies have shown that digestion with a protease in the presence of denaturing agents is sufficient to eliminate the infectivity of prion (22), others suggested a difference between the removal of immunoreactive material and infectivity (23-27). Alternatively, residual infectivity may be found in the portion of PrP^{Sc} not recognized by SAF83. Therefore, a mouse bioassay of infectivity would be needed to test the effectiveness of these commercial alkaline detergents and proteases.

Taken together, the results of this study demonstrate the usefulness of commercial alkaline detergents and proteases for the degradation or destabilization of PrP^{Sc}, and suggest that their effectiveness against prion can be increased.

References

1. Prusiner SB: Prions. Proc Natl Acad Sci USA 95: 13363-13383, 1998.
2. Sakudo A and Ikuta K: Prion protein functions and dysfunction in prion diseases. Curr Med Chem 16: 380-389, 2009.
3. Ramasamy I, Law M, Collins S and Brooke F: Organ distribution of prion proteins in variant Creutzfeldt-Jakob disease. Lancet Infect Dis 3: 214-222, 2003.
4. Sakudo A, Onodera T, Suganuma Y, Kobayashi T, Saeki K and Ikuta K: Recent advances in clarifying prion protein functions using knockout mice and derived cell lines. Mini Rev Med Chem 6: 589-601, 2006.
5. Sakudo A and Ikuta K: Fundamentals of prion diseases and their involvement in the loss of function of cellular prion protein. Protein Pept Lett 16: 217-229, 2009.
6. Armstrong RA: Creutzfeldt-Jakob disease and vision. Clin Exp Optom 89: 3-9, 2006.
7. Rutala WA and Weber DJ: Creutzfeldt-Jakob disease: recommendations for disinfection and sterilization. Clin Infect Dis 32: 1348-1356, 2001.
8. McDonnell G and Burke P: The challenge of prion decontamination. Clin Infect Dis 36: 1152-1154, 2003.
9. Walker JT, Dickinson J, Sutton JM, Marsh PD and Raven ND: Implications for Creutzfeldt-Jakob disease (CJD) in dentistry: a review of current knowledge. J Dent Res 87: 511-519, 2008.
10. Peden AH, Head MW, Ritchie DL, Bell JE and Ironside JW: Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet 364: 527-529, 2004.
11. Wroe SJ, Pal S, Siddique D, *et al*: Clinical presentation and premortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. Lancet 368: 2061-2067, 2006.
12. World Health Organization: WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies: Report of a WHO Consultation Geneva. World Health Organization, Stationery Office Books, Switzerland, 2001.
13. Brown SA, Merritt K, Woods TO and Busick DN: Effects on instruments of the World Health Organization - recommended protocols for decontamination after possible exposure to transmissible spongiform encephalopathy-contaminated tissue. J Biomed Mater Res B Appl Biomater 72: 186-190, 2005.
14. Baier M, Schwarz A and Mielke M: Activity of an alkaline 'cleaner' in the inactivation of the scrapie agent. J Hosp Infect 57: 80-84, 2004.
15. Lemmer K, Mielke M, Pauli G and Beekes M: Decontamination of surgical instruments from prion proteins: in vitro studies on the detachment, destabilization and degradation of PrP^{Sc} bound to steel surfaces. J Gen Virol 85: 3805-3816, 2004.
16. Sakudo A, Wu G, Onodera T and Ikuta K: Octapeptide repeat region of prion protein (PrP) is required at an early stage for production of abnormal prion protein in PrP-deficient neuronal cell line. Biochem Biophys Res Commun 365: 164-169, 2008.
17. Barret A, Tagliavini F, Forloni G, *et al*: Evaluation of quinacrine treatment for prion diseases. J Virol 77: 8462-8469, 2003.
18. McLeod AH, Murdoch H, Dickinson J, *et al*: Proteolytic inactivation of the bovine spongiform encephalopathy agent. Biochem Biophys Res Commun 317: 1165-1170, 2004.
19. Langeveld JP, Wang JJ, Van de Wiel DF, *et al*: Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. J Infect Dis 188: 1782-1789, 2003.
20. Hui Z, Doi H, Kanouchi H, *et al*: Alkaline serine protease produced by Streptomyces sp. degrades PrP(Sc). Biochem Biophys Res Commun 321: 45-50, 2004.
21. Fichet G, Comoy E, Duval C, *et al*: Novel methods for disinfection of prion-contaminated medical devices. Lancet 364: 521-526, 2004.
22. Caughey B, Raymond GJ, Kocisko DA and Lansbury PT Jr: Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies. J Virol 71: 4107-4110, 1997.
23. Rubenstein R, Carp RI, Ju W, *et al*: Concentration and distribution of infectivity and PrPSc following partial denaturation of a mouse-adapted and a hamster-adapted scrapie strain. Arch Virol 139: 301-311, 1994.
24. Wille H, Zhang GF, Baldwin MA, Cohen FE and Prusiner SB: Separation of scrapie prion infectivity from PrP amyloid polymers. J Mol Biol 259: 608-621, 1996.
25. Fernandez Garcia A, Heindl P, Voigt H, *et al*: Reduced proteinase K resistance and infectivity of prions after pressure treatment at 60 degrees C. J Gen Virol 85: 261-264, 2004.
26. Yao HL, Han J, Gao JM, *et al*: Comparative study of the effects of several chemical and physical treatments on the activity of protease resistance and infectivity of scrapie strain 263K. J Vet Med B Infect Dis Vet Public Health 52: 437-443, 2005.
27. Heindl P, Fernandez Garcia A, Buttner M, *et al*: Some physicochemical parameters that influence proteinase K resistance and the infectivity of PrPSc after high pressure treatment. Braz J Med Biol Res 38: 1223-1231, 2005.