

Enzymatic Degradation of Prion Protein in Brain Stem from Infected Cattle and Sheep

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Prions—contagious agents involved in transmissible spongiform encephalopathies—normally survive proteolytic and mild protein-destructive processes. Using bacterial keratinase produced by *Bacillus licheniformis* strain PWD-1, we tested conditions to accomplish the full degradation of prion protein (PrP) in brain-stem tissue from animals with bovine spongiform encephalopathy and scrapie. The detection of PrP^{Sc}, the disease-associated isoform of PrP, in homogenates was done by Western blotting and various antibodies. The results indicated that only in the presence of detergents did heat pretreatment at >100°C allow the extensive enzymatic breakdown of PrP^{Sc} to a state where it is immunochemically undetectable. Proteinase K and 2 other subtilisin proteases, but not trypsin and pepsin, were also effective. This enzymatic process could lead to the development of a method for the decontamination of medical and laboratory equipment. The ultimate effectiveness of this method of prion inactivation has to be tested in mouse bioassays.

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders of humans and animals. In clinical cases, infectivity is accompanied by the conversion of the host-encoded prion protein PrP^C into a structurally altered form, PrP^{Sc}. No differences in primary structure have been found between PrP^C and PrP^{Sc} [1]. Although PrP^C occurs in both healthy and diseased tissues, the presence of PrP^{Sc} is typical for the diseased state and is biochemically detectable as PrP^{res}. PrP^{Sc} has a high content of β -sheet conformation, is partially resistant to proteinase K digestion, and is believed to exist as an aggregate [2, 3]. In the presence of detergents, PrP^{Sc} and infectivity can be effectively recovered from tissue homogenates at high centrifugal force, although the relationship between the 2 has not yet been clearly defined [4, 5].

The infective agent is highly resistant to treatments that destroy nucleic acids and is mainly, if not completely, composed of PrP^{Sc} [6]. In practice, contaminated materials are usually disinfected by boiling in 1% SDS under reducing conditions, boiling in 1 mol/L sodium hydroxide, or treatment with formic acid or bleach containing 20,000 ppm active chlorine [7–9]. These are harsh conditions. More compatible alternatives would be preferred, if possible. Strains of the agent differ in their resistance to heat inactivation, and the bovine spongiform encephalopathy (BSE) agent is one of the more resistant examples [9–13]. In addition to the conventional techniques mentioned, combined treatments of denaturation, heat, and/or high pressure are under investigation [16, 17]. Therefore, additional ways to inactivate BSE and other TSE agents would be highly desirable. They would be especially useful for the treatment of valuable medical instruments, laboratory equipment, and exchangeable items like contact lenses and dentistry tools [14, 15].

Decontamination based on enzymatic breakdown would offer an attractive possibility either as a single method or in combination with other inactivation treatments. Treatments that disrupt hydrogen bonds and favor solubilization would allow better degradation by proteases. Furthermore, the type of enzyme and di-

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gestion conditions are of crucial importance to obtain a high degree of inactivation. It has been found elsewhere that the proteolytic treatment of crude or partially purified scrapie from infected mouse and hamster brain poorly neutralizes infectivity [18–20], whereas, in purified hamster scrapie, proteinase K is capable of reducing infectivity by >4 orders of magnitude, although a considerable level of residual infectivity remained [20, 21]. For purified hamster scrapie material, pretreatment with heat, denaturants, or high pH made PrP^{Sc} [22] and infectivity highly susceptible to degradation by proteinase K [23]. By contrast, in crude homogenates of mouse brain, heat inactivation did not always lead to the complete protease sensitivity of PrP^{Sc}, although infectivity was destroyed [13]. It is possible that PrP^{Sc} in tissue may be more protected than in purified prion preparations from heat denaturation and protease degradation or that the protease used was not effective. Because solubilization, heat, and enzymatic digestion are physically different processes, the combined effect of each could result in enhanced inactivation.

Conditions were investigated to obtain a high-level degradation of PrP^{Sc} by a combined treatment of solubilization, heat denaturation, and enzymatic digestion. This was performed with tissue homogenates of BSE- and scrapie-infected brain stem. Because of its broad substrate proteolytic activity, the enzyme used for digestion was a feather-degrading keratinase, which was recently isolated from *Bacillus licheniformis* PWD-1 (PWD-1 keratinase) and has been well characterized and produced on a large scale [24, 25].

MATERIALS AND METHODS

Tissues. All activities were carried out in a pathogen class-3 facility, in agreement with European Union directives and the guidelines set out by the Spongiform Encephalopathy Advisory Committee of the United Kingdom. Brain-stem material was derived from adult cattle and sheep with or without clinical signs of disease. A diagnosis of BSE and scrapie was made on samples from the obex region by 3 methods for different characteristics: histopathology for vacuoles [26], immunohistochemical techniques for the deposition of PrP [27], and on homogenates for detection of PrP^{res} by Western blotting with the Prionics Check method [28].

Enzymes and determination of protease activity. PWD-1 keratinase was produced and purified as described elsewhere [24, 25]. Proteinase K from *Tritirachium album* was purchased as purified crystalline product from Merck. The following enzymes were all purchased from Sigma Chemical as purified and crystalline material: *Clostridium histolyticum* collagenase type VII, hog pancreas elastase type III, hog stomach pepsin, bovine pancreas trypsin, *B. licheniformis* alcalase, and *Bacillus subtilis* subtilisin Carlsburg. The protein substrates bovine tendon collagen (Sigma type V), bovine neck ligament elastin, and bovine

casein were all purchased as powder from Sigma Chemical. Feather keratin was prepared by grinding and ball milling, as described elsewhere [24].

All enzyme activities were measured at their individual optimum conditions (according to Sigma Chemical manuals). The hydrolysis rate of keratin, elastin, and collagen was measured by a ninhydrin color reaction (absorbance at 590 nm) of increased free amino groups [24]. The casein hydrolysis rate was measured by the increased absorbance at 280 nm in the supernatant [29]. Relative activities of individual proteases were compared and calculated for each substrate, using as unity the average value obtained with the related specific enzyme (i.e., keratinase on keratin, elastase on elastin, etc.).

Tissue treatment. Pieces of 0.3–0.6 g of brain-stem material, cut from within 5 cm of the obex region were homogenized with an Omni International TH mixer with a disposable probe in 10× vol of 2% (wt/vol) *N*-lauroylsarcosine (Sigma) in 0.1 mol/L sodium phosphate (pH 7.2; lysis buffer). When specified, *N*-lauroylsarcosine was replaced by a mixture of 2 detergents, 0.5% sodium deoxycholate and 0.5% Triton X-100 in 50 mmol/L Tris-HCl. In some experiments, tissue was first cut into small blocks, divided in 2 aliquots, and either homogenized in lysis buffer or first heated before homogenization in lysis buffer. The heating of homogenates was performed for 40 min at 115°C (pressure, 72 kPa) in a Vulcan pressure cooker, unless specified otherwise. The escape of aerosols from the vials was prevented by sterilization stoppers made of filter paper. In experiments to evaluate the effect of different temperatures <115°C, homogenates were heated for 40 min in 2-mL screw-cap vials in a temperature-controlled heating block with tightly fitting holes. Homogenates were stored at 4°C–8°C unless otherwise specified.

Enzymatic digestion of brain-stem homogenates. For PWD-1 keratinase, digestions were typically done at 50°C for 1 h with 100 µL of tissue homogenate and 10 µL of purified enzyme at a final concentration of 200 µg/mL in a 96-well polypropylene digestion plate designed for polymerase chain reaction. Reactions were stopped by the addition of 10 µL of 12.5 mmol/L Pefabloc SC (Roche). Digestions with other proteases were done under the same conditions, except that temperature of incubation was different depending on the enzyme used: proteinase K, 50°C; alcalase and subtilisin Carlsburg, 35°C; and trypsin and pepsin, 37°C. In the case of pepsin, the homogenate was first adjusted to pH 2–3 by the addition of 4 µL of 0.1 mol/L HCl, and, at the end of the digestion, the pH was readjusted to pH 7–8 by the addition of 8 µL of 1 mol/L Tris.

Gel electrophoresis, Western blotting, and immunodetection of PrP. If not specified, all samples were precipitated by the addition of 9× volume of methanol and subsequent centrifugation. Pellets were subsequently taken up by thorough trituration in 100 µL of sample buffer, as described in a manual

for NuPage gels from Invitrogen. Electrophoresis in SDS-PAGE, the Western blotting procedure, PonceauS staining for total protein, and immunostaining with PrP-specific antibodies were done according to established procedures [28, 30–32]. Prion protein was detected with primary antibody 6H4 [33] at 0.2 μg IgG/mL and rabbit anti-mouse IgG antibody conjugated to alkaline phosphatase (DAKO). In some cases, additional PrP detection was done with other PrP-specific antibodies at concentrations that would warrant the detection of traces and various epitopes of bovine PrP antigen: rabbit polyclonal antibody R505 (raised with ovine PrP peptide 100–111) at 1:2000 dilution, and 4 different murine monoclonal antibodies each at 1 μg /mL: 6H4, 66.94b4 (raised at Central Institute for Animal Disease Control, Lelystad, The Netherlands, with human and bovine recombinant PrP, which binds to a conformational epitope of the proteinase K-resistant part of PrP^{Sc}), F99/97.6.1 (raised with ovine PrP peptide 217–231), and 12F10 (raised with DNA coding for human PrP; it binds to synthetic peptide 141–159 from the human PrP sequence; SPI-BIO) [34–37]. The development of the signal was done with CDP-Star, according to the supplier's instructions (Tropix), and signals were recorded on photographic film (Eastman Kodak). In addition to molecular-weight markers, 2 controls were used. First, a BSE-infected brain stem was homogenized and, without heat pretreatment, digested with proteinase K, according to the Prionics Check protocol (sample P). This showed the migration of PrP^{res}, the typical protease-resistant fraction of PrP^{Sc}, which consists of a diglycosylated, monoglycosylated, and unglycosylated protein band. Second, a nondigested brain-stem homogenate from a healthy cow (sample N) served as a control, to show the migration of intact PrP^C and PrP^{Sc}. There was no difference seen in the migrational behavior between these 2 isoforms when they were left intact. Molecular-weight markers, mixed with the control, were carbonic anhydrase from bovine erythrocytes (29 kDa; Sigma) and β -lactoglobulin from bovine milk (18.4 kDa; Sigma). In some experiments, the density of complete (top to bottom) lanes was recorded from the film with an Agfa Duo T200XL scanner and further processed with GelPro software.

RESULTS

General comparison of different proteases with PWD-1 keratinase. Purified PWD-1 keratinase from *B. licheniformis* PWD-1 was tested for its general capacity to break down proteins known to be poorly hydrolyzed by proteases. PWD-1 keratinase exhibited excellent proteolytic properties in digesting keratin, elastin, collagen, and casein, compared with other proteases like elastase, collagenase, proteinase K, and trypsin (data not shown). PWD-1 keratinase was therefore further used for the prion-degradation studies.

Degradation of PrP^{Sc} by keratinase in cattle and sheep tissue.

It appeared that, when tested on BSE-infected brain-stem material homogenates that were not heat treated, PWD-1 keratinase, like proteinase K, was not capable of degrading the PrP^{Sc} beyond the typical PrP^{res} product, with a banding pattern of diglycosylated, monoglycosylated, and unglycosylated PrP forms (figure 1A, sample P and lanes 13–16). Prion protein in negative material was fully digested. However, when the BSE-positive homogenates were preheated at 115°C in a pressure cooker, PWD-1 keratinase fully abolished the immunodetectability of PrP^{Sc} (figure 1A, lanes 9–12). It appeared that the distribution of PrP^{Sc} can vary between the soluble and insoluble fractions, especially after the freezing of brain homogenates; this possibly could influence the resistance to proteolytic degradation. Therefore, we tested whether all PrP was digested from both whole homogenate and the soluble fraction only, both in unfrozen and frozen aliquots. Indeed, PrP^{Sc} degradation by keratinase was effective for both whole homogenates, the supernatant, and frozen aliquots thereof (e.g., figure 1A, lanes 9–12). This high extent of proteolytic degradation was not only established with antibody 6H4 at a high concentration (1 μg /mL) but also with 4 additional antibodies, R505, 66.94b4, 12F10, and F99/97.6.1, which have different specificities for PrP^{res} (data not shown). Few coagulates were seen in the vials after heating in the presence of detergent, and, possibly as a result, only in nondigested samples was immunoreactive material observed at the top of gel lanes. A PrP-positive protein with properties of PrP^C was observed when the enzyme was omitted from the digestion mixture, which indicates that most of the PrP protein was dissociated in its monomeric form in the samples applied to the gel (figure 1B, lanes 3–4; figure 1C, lanes 1–2). These experiments were performed in the presence of 2% (wt/vol) of the detergent *n*-lauroylsarcosine. To obtain full digestion, the presence of the detergent was essential. Protease-resistant PrP^{Sc} remained detectable in BSE-positive homogenates when heating was carried out in the absence of detergent, whereas normal PrP was fully digestible in BSE-negative tissue (figure 1B, lanes 1–2 lanes 5–6). Different types of detergent used in the homogenate during the heating step had different effects on the extent of subsequent digestion. When 2% *n*-lauroylsarcosine was used, PrP^{Sc} was fully digested; when a mixture of Triton X-100 and sodium deoxycholate was used, residual PrP^{res} was observed (figure 1C, lanes 3–4). Effective digestion after preheating in the presence of *n*-lauroylsarcosine occurred in all samples tested: BSE- and scrapie-infected brain stems from cattle and sheep, respectively (figure 2).

Optimization of PrP^{Sc} digestion with keratinase. PWD-1 keratinase at a concentration >10 μg /mL, digested PrP for 60 min to levels below detection, whereas, in comparison, proteinase K was somewhat more active, yielding such results at enzyme concentrations >5 μg /mL, compared with proteinase K (figure 3A). Under suboptimal enzyme concentrations like 10 μg /mL,

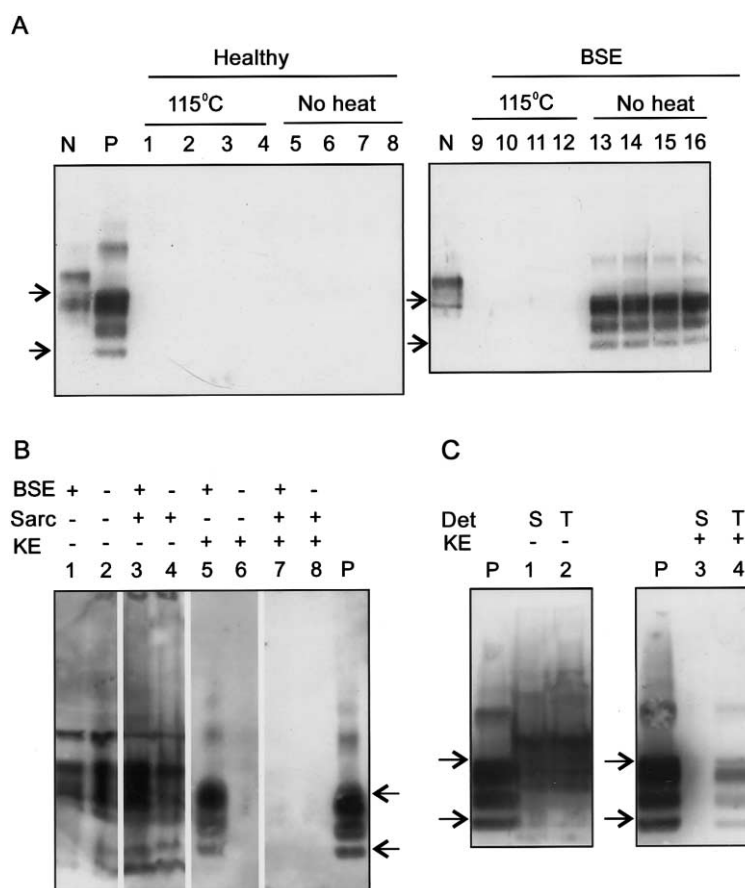


Figure 1. Effect of heat pretreatment and detergent on the digestion of prion protein (PrP)^{Sc} by keratinase. *A*, Brain-stem tissue from healthy and bovine spongiform encephalopathy (BSE)-infected cow were pretreated at 115°C. Different aliquots after the heat pretreatment were differently processed: whole homogenates (lanes 1, 2, 5, 6, 9, 10, 13, and 14), supernatants collected from centrifugation at 2000 *g* for 15 min (lanes 3, 4, 7, 8, 11, 12, 15, and 16), and aliquots frozen for 17 days (lanes 2, 4, 6, 8, 10, 12, 14, and 16). *B*, The effect of preheating in the presence of N-lauroylsarcosine (sarc) on subsequent digestion with keratinase (KE) is shown. Samples were not precipitated with methanol. *C*, The effect of detergents is shown. Before heating, homogenates from a BSE-infected animal were prepared in 2 different lysis buffers with different detergent compositions: 2% N-lauroylsarcosine (S) vs. 0.5% Triton X-100 and 0.5% sodium deoxycholate (T). Material was studied both without and after digestion with KE. The tissue equivalents applied were 1000 μ g, except 200 μ g in lane N of panel A and 400 μ g in all lanes of panel B. Arrows indicate the position of molecular-weight markers carbonic anhydrase (29 kDa) and β -lactoglobulin (18.4 kDa). All homogenates were prepared in the presence of 2% N-lauroylsarcosine, except where indicated. See Materials and Methods for full details. +, Infected with BSE; –, healthy; N, sample containing nonheated nondigested brain-stem material from a healthy cow (control for intact PrP); P, unheated sample from a BSE-infected cow digested with proteinase K (PrP^{res} control).

the degrading activity of PWD-1 keratinase continued for ~30–60 min. However, at this low enzyme concentration and at longer incubation times (tested up to 4 h), the signal of intact PrP did not completely disappear. Surprisingly, this was also the case for BSE-negative tissue, which indicates that the protease activity for tissue proteins was exhausted (figure 3*B*). The molecular-weight pattern of the residual PrP in the samples digested at these low enzyme concentrations corresponded with that of PrP^C in non-digested material (data not shown). Thus, for subsequent studies, a concentration of 200 μ g/mL protease in 10% tissue homogenates and incubation time for 1 h were considered to be conditions sufficient for the full degradation of PrP^{Sc}.

Varying the temperature during heat pretreatment of tissue

homogenates. The digestion by PWD-1 keratinase might be effective in homogenates when preheated at temperatures <115°C. This was investigated for both scrapie- and BSE-infected brain-stem homogenates at temperatures between 70°C and 115°C (figure 4). Heating homogenates at 70°C–90°C hardly led to the susceptibility of PrP^{Sc} to digestion by keratinase. Heating at 100°C led to a significant reduction in the PrP^{res} signal (45%–65% of the value obtained in material heated at 70°C). However, only at autoclaving conditions (115°C) were PrP^{res} signals reduced to undetectable levels.

Comparison of different proteases for their capacity to degrade PrP^{Sc}. Some other purified proteases were also tested. At a concentration of 200 μ g/mL and their respective optimum

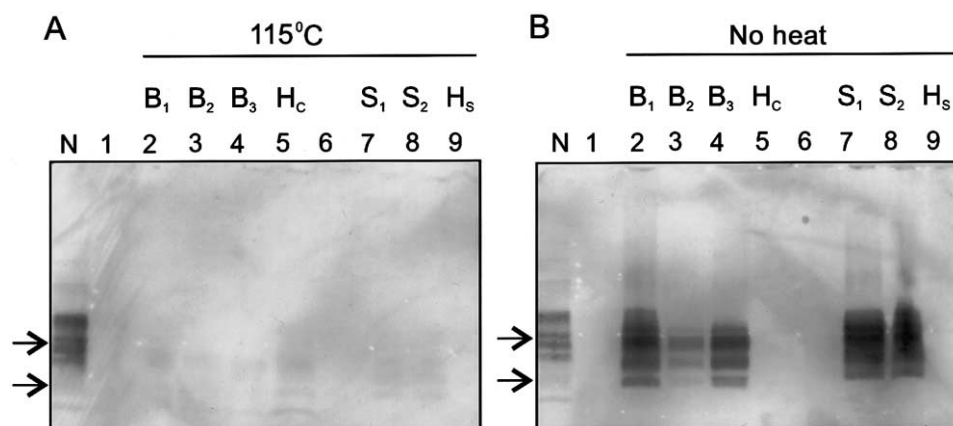


Figure 2. Digestion with keratinase of heated and unheated bovine spongiform encephalopathy (BSE) and scrapie samples from different individual animals is shown. Heat pretreatment is indicated. None of the samples was precipitated with methanol prior to loading. B₁, B₂, and B₃, samples from 3 different BSE-infected cattle; H_c and H_s, samples from healthy cow and sheep, respectively; N, sample containing nonheated nondigested brain-stem material from a healthy cow (control for intact PrP); S₁ and S₂, samples from 2 different scrapie-infected sheep. Lanes 1 and 6 are empty. Tissue equivalents applied: 400 μ g, except lane N, 200 μ g. Arrows indicate 29- and 18.4-kDa molecular-weight markers.

temperature and pH, proteinase K, alcalase, and subtilisin Carlsburg were able to fully degrade PrP from heated brain-stem homogenates, whereas, without heating, the typical PrP^{res} pattern appeared for each of these enzymes (figure 5). PrP^{res} digestion with trypsin (and pepsin at pH 2.4 and 37°C; data not shown) were incomplete on heated samples; hardly any degradation occurred on unheated material.

DISCUSSION

The present study demonstrates that it is possible to destroy PrP^{Sc} in infected crude brain material toward immunochemically undetectable levels. The theoretical basis for the result is that the aggregated and β -sheeted structure of the rogue PrP protein was first denatured by heat in the presence of detergent and subsequently digested with a broad-spectrum serine endopeptidase. The case is similar to the keratinase degradation of feather keratin, which is also rich in β -sheet structure. In addition to PWD-1 keratinase, proteinase K and 2 proteases of the subtilisin family were found to be capable of degrading PrP. It is assumed that this method can reduce infectivity at least 3 orders of magnitude and has the potential to accomplish complete inactivation.

The effectiveness of the method was monitored by Western-blot assay, a test that is dependent on the detection by PrP-specific antibodies and recovery of the BSE agent (i.e., the solubility of the material). Recently, immunochemical tests for the detection of PrP^{Sc}, like Western blotting, have been found to be suitable for the routine diagnosis of BSE in the brain-stem tissue of cattle [28, 38]. The sensitivity of such immunochemical tests appears to be comparable with or better than bioassays with unpassaged BSE material [39]. The limitation of this comparison, however, should be kept in mind. BSE testing in conventional

mice is relatively insensitive, reaching variable titers in the range of $10^{2.7}$ – $10^{5.2}$ ID₅₀/g of brain tissue [40, 41]. More-sensitive test systems for unpassaged BSE have been developed in transgenic mice that express multiple copies of the bovine PrP gene. These systems reach titers of 10^7 – 10^8 ID₅₀/g of brain-stem tissue [42, 43]. For sheep scrapie, however, such a reproducible and high-titer mouse bioassay is still not available.

The combination of denaturation by heat and subsequent enzymatic treatment to destroy PrP^{Sc} is potentially an adaptable procedure to eliminate prions from contaminated equipment. Harsh chemicals are not involved in this method. The presence of a detergent like *N*-lauroylsarcosine is required for the full digestion of PrP^{Sc}, presumably because the detergent facilitates both its solubilization and denaturation during the heating pretreatment. For surface cleaning, these are normal conditions in hospital and laboratory equipment. For the production of food proteins from animal byproducts, however, low-cost natural substitutes for the detergent should be sought. Heating >80°C is indispensable for the elimination of any microbial organisms. To obtain full proteolytic susceptibility, only preheating at 115°C was effective in the presence of *N*-lauroylsarcosine, and this temperature provided less-satisfactory results with the weaker detergents Triton X-100 and sodium deoxycholate, in which some PrP^{res} remained after subsequent digestion with keratinase. Potential improvements are to be expected in the future by a balanced regimen of denaturing temperature, choice of detergent, and enzymatic digestion. However, a safe temperature for pretreatment (115°C) to obtain an optimal enzymatic degradation of PrP^{res} is, in fact, a temperature that even is too high for potential proteases from thermophilic microorganisms.

The infectivity and protease resistance of PrP^{Sc} present themselves as 2 different properties of the TSE agent, and the re-

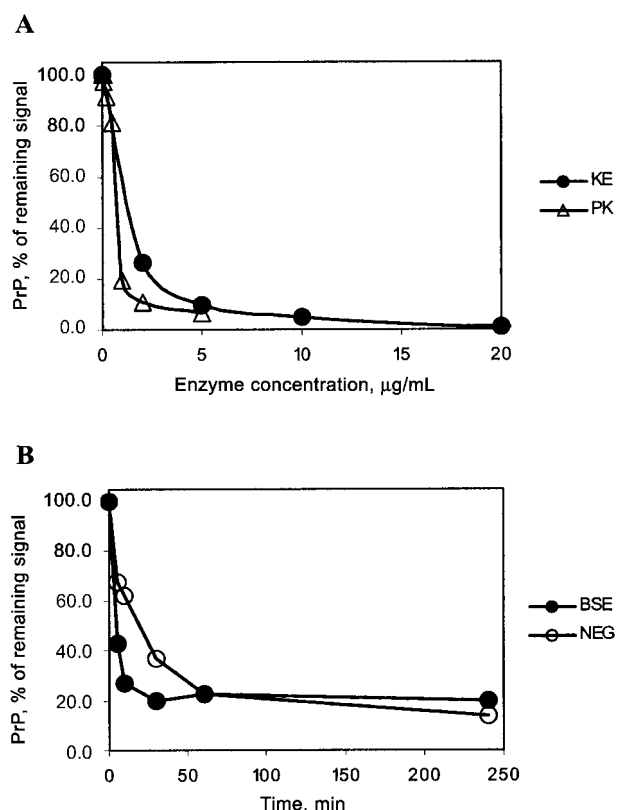


Figure 3. The effects of concentration and incubation time on keratinase (KE) digestion of prion protein (PrP) in heated brain-stem homogenate are shown. *A*, Bovine spongiform encephalopathy (BSE)-infected brain-stem homogenates digested at variable KE and proteinase K (PK) concentrations during a fixed incubation time of 60 min. *B*, The extent of digestion of PrP in BSE-positive (BSE) and -negative (NEG) tissue after varying time of incubation. Aliquots of 10% brain-stem homogenate were digested by KE PWD-1 at suboptimal concentration of 10 µg/mL. For both panels, photographic films of Western blots were recorded by a scanner, and the resulting densities further processed with GelPro software. Each data point is an integrated recording of a complete lane and is expressed as the percentage of the density signal obtained for 0 time incubation samples.

relationship between PrP^{Sc} and in vivo infectivity has not been clearly defined. Somerville et al. [13] reported that, in mouse brain homogenates infected with either of 2 adapted scrapie strains, ME7 and 22C, proteinase K-resistant PrP^{Sc} survived heating in the absence of detergent, although infectivity was reduced by >99.9%. The favorable use of detergent during heating to improve the proteolysis of PrP^{Sc} was shown: protease-resistant PrP^{Sc} remained when heating was done in the absence of detergent (figure 1*B*), but PrP^{Sc} was fully susceptible to keratinase when detergent was present. We postulate that, when homogenates are treated according to the presented procedure using effective detergents for extraction combined with proteolysis, infectivity like that of PrP^{Sc} will be more effectively destroyed than when it is heated in the absence of detergent.

It could be that the agent, if it is completely composed of PrP^{Sc}, would exist in at least 2 different multimeric β -sheeted forms—one an unaggregated structure and the other as an aggregated fraction—like amyloid with Congo red affinity [44]. Although both forms are infective and the infectivity is most susceptible to heat, their proteolytic susceptibility is dependent on the deaggregated and denatured state caused by heat in the presence of detergent. Proper denaturation during heating might be obtained only in the presence of an effective detergent (e.g., SDS or sarcosyl) or possibly other chaotropic conditions common in protein research (e.g., low pH or the use of urea or guanidium).

PrP^{Sc} in (nondenatured) brain homogenates from infected animals can be partially digested by proteinase K such that the characteristic conversion occurs of PrP^C into PrP^{Res} [45, 46]. This molecular-weight transition has been a long-standing benchmark for the presence of PrP^{Sc}. Our data show that other proteases, such as subtilisins and PWD-1 keratinase but not trypsin and pepsin, also have this capacity. In addition, keratinase, proteinase K, and the 2 other subtilisins, although they are enzymes from different sources and with different amino-acid sequences, also yield the same-sized PrP^{Res} bands. This phenomenon of a similar end result of proteolysis might be based on the conformational properties of PrP^{Sc} that determine the extent of its digestion for these serine endopeptidases.

The present study is a biochemical test that shows an effective erasure of prion protein. It promises new avenues for a decontamination or inactivation process, which is highly needed [47, 48]. Of basic significance, however, is the in vivo bioassay. BSE from cattle or as propagated from mice has been found to be resistant to heat inactivation [10, 11]. Experiments are in prep-

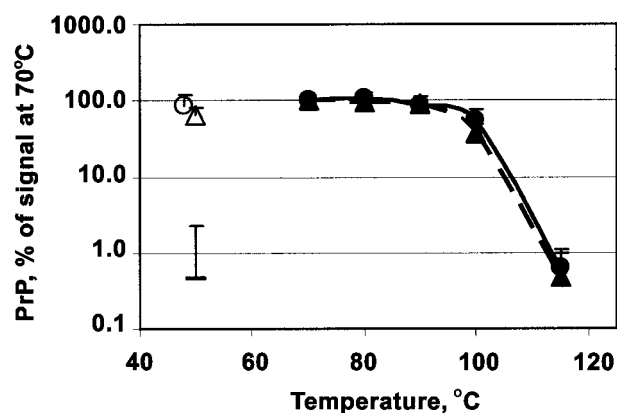


Figure 4. Different temperatures during the heat pretreatment of tissue homogenates from a bovine spongiform encephalopathy (BSE)-infected cow and a scrapie-infected sheep are shown. Data points represent average values from 3 digestion experiments plus SD and are expressed on the logarithmic axis as the percentage of the prion protein (PrP) signal obtained with the sample preheated at 70°C. White symbols, not preheated; black symbols, preheated at the temperature indicated; circles, BSE infected; triangles, scrapie infected; bar, a sample from healthy cow.

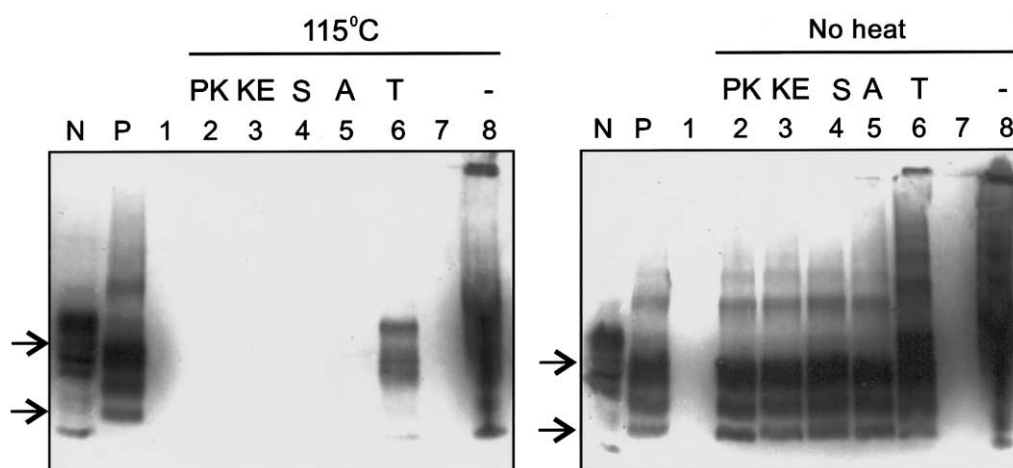


Figure 5. Digestion of prion protein (PrP)^{Sc} in bovine spongiform encephalopathy (BSE)-infected brain-stem homogenates by various enzymes: A, alcalase; KE, PWD-1 keratinase; PK, proteinase K; S, subtilisin Carlsburg; and T, trypsin. Heat pretreatment is indicated. The tissue equivalents applied were 1000 μ g/lane, except for lane N, 200 μ g. Lanes P and N are controls, as described in figure 1 and Materials and Methods. Lanes 8, no enzyme; lanes 1 and 7, empty.

aration to verify the biological effectiveness of this method with BSE field isolates and with the high-titer BSE strain 301V in mice. These experiments require 2–3 years to obtain the answer. It is, however, of general importance that transgenic mice and in vitro cellular assays will become generally available for the testing of unpassed TSE agents with respect to BSE, scrapie, chronic wasting disease, and Creutzfeldt-Jakob disease. Such assays ideally need to be sensitive, flexible toward the testing material, and of relatively short duration.

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