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Some physico-chemical parameters that influence proteinase K resistance and the infectivity of PrP^{Sc} after high pressure treatment

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Abstract

Crude brain homogenates of terminally diseased hamsters infected with the 263 K strain of scrapie (PrP^{Sc}) were heated and/or pressurized at 800 MPa at 60ºC for different times (a few seconds or 5, 30, 120 min) in phosphate-buffered saline (PBS) of different pH and concentration. Prion proteins were analyzed on immunoblots for their proteinase K (PK) resistance, and in hamster bioassays for their infectivity. Samples pressurized under initially neutral conditions and containing native PrP^{Sc} were negative on immunoblots after PK treatment, and a 6-7 log reduction of infectious units per gram was found when the samples were pressurized in PBS of pH 7.4 for 2 h. A pressure-induced change in the protein conformation of native PrP^{Sc} may lead to less PK resistant and less infectious prions. However, opposite results were obtained after pressurizing native infectious prions at slightly acidic pH and in PBS of higher concentration. In this case an extensive fraction of native PrP^{Sc} remained PK resistant after pressure treatment, indicating a protective effect possibly due to induced aggregation of prion proteins in such buffers.

Key words
- Native prion protein
- Pressure
- Inactivation
- pH
- Proteinase K sensitivity
- Prion conformation

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Introduction

Prions cause neurodegenerative diseases such as scrapie in sheep, bovine spongiform encephalopathy in cattle, Creutzfeld-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia, kuru, and a new variant of Creutzfeld-Jakob disease in humans (1,2). These transmissible spongiform encephalopathies are associated with the accumulation of a misfolded form (PrP^{Sc}) of the cellular prion protein (PrP^{C}) in brain. The misfolded ß-sheet-rich aggregated pathogenic multimer seems to be the main component of the transmissible form and the infectious agent (3). The unconventional agents that cause prion diseases are known to be relatively resistant to a wide variety of inactivation procedures that are effective with microorganisms (4-6). Procedures such as exposure to 1 M sodium hydroxide for 1 h at room temperature, gravity-displacement autoclaving at 132ºC for 1 h, or porous-load autoclaving at 134-138ºC for 18-60 min do not provide complete inactivation (7). Nevertheless, the use of sodium hypochlorite solutions containing at least 20,000 ppm of

available chlorine (5) or the combination of autoclaving at 121ºC and sodium hydroxide exposure (6) are effective methods for a complete inactivation of prions. All these suitable treatments are aggressive, with a consequent loss of quality and texture in the treated tissues. Therefore, our interest in assessing the effects of unconventional milder technologies on prion stability and prion infectivity arises from the necessity of providing alternative sterilization procedures for risk materials.

High hydrostatic pressure is a mild processing technology with a promising potential for food pasteurization and sterilization. Undesirable alterations in foods, such as vitamin loss and changes in taste and color can be minimized by avoiding heat sterilization (8,9). Moreover, ultra-high pressure-assisted thermal sterilization of infectious prion proteins has already been reported to be effective when the temperature is shifted up to 135-142ºC (10). This emerging food processing technology exploits the unique effect of adiabatic heating and cooling. In addition, high pressure is a thermodynamic parameter providing useful information when studying protein denaturation and reaction mechanisms (11). High hydrostatic pressure shifts equilibrium and accelerates processes for which the transition state has a smaller volume than the ground state. Thus, unexpected effects, different from temperature or chemical denaturation, can be seen under pressure (12). Pressure induces volume changes of the protein solvent system, which is associated with conformational changes, protein folding/unfolding and aggregation. Its largest contribution is at the level of weak non-covalent interactions, while hydrogen bonds, which stabilize the secondary structure of the protein, are hardly affected under pressure, and hydrophobic and electrostatic interactions are disfavored. Therefore, the quaternary structure of the protein is usually the most sensitive to pressure (13).

Mild high pressure treatments induce the conversion of the non-infectious cellular prion protein to a proteinase K (PK)-resistant form (14,15). Additionally, a satisfactory reduction in the PK resistance and an impressive reduction in the infectivity of hamster prion proteins have been reported with pressurization at temperatures below customary sterilization parameters (16). This observed irreversible pressure inactivation of the infectious prion protein is unexpected and remarkable since usually only extreme high pressures are able to rearrange the secondary structures of proteins and hydrogen bridges and ß-sheets should remain resistant to such high pressure treatments.

We report here on the effects of a treatment with high hydrostatic pressure at 800 MPa and 60ºC with varying pressure-holding times and buffer pH on PK sensitivity and the *in vivo* infectivity of hamster PrP^{Sc} in phosphate-buffered saline (PBS). Results were expected to be useful as a basis for the application of high pressure as an alternative technology for the mild inactivation of infectious prions at temperatures that avoid thermal damage.

Material and Methods

Brain homogenates

Experiments with native PrP^{Sc} were performed using different brain pools of the 263 K strain of the hamster-adapted scrapie agent. Brains from terminally diseased hamsters containing PrP^{Sc} were homogenized in a 1:10 PBS dilution (1-fold concentrated: 8 mM phosphate buffer, 120 mM NaCl, 27 mM KCl), pH 5.6, 6.4, 7.4, 7.9 in a FastPrep cell disrupter (Qbiogene, Illkirch, France). Sets of duplicate samples were each heated at 60ºC or pressurized at 800 MPa and 60ºC independently.

High pressure treatments

Total volumes of 250 µl hamster brain homogenate (10^{-1}, w/v) were pressurized in

a hydraulic press U 101 (Polish Academy of Sciences, Warsaw, Poland). U 101 is a manually operated hydraulic press (100 mm piston length and 80 mm piston movement). The vessel is 150-mm high cylinder made of steel, with an inner diameter of 16 mm. The piston position is monitored with a linear transformer transducer and the pressure-measuring unit is an in-vessel manganin pressure gauge; both are digitally displayed. The pressure-transmitting medium was a 7:3 mixture of petroleum ether (boiling point 80-100ºC) and hydraulic oil (SAE 32). Polyethylene caps were perfectly isolated in heat-sealed polyethylene-coated aluminum bags before pressurization.

The effect of adiabatic heating was minimal due to the long pressure rise necessary to achieve the highest pressures (at least 120 s for 800 MPa) and to the continuous control of the temperature in the vessel using a thermostat (Polystat, Huber, Germany) coupled to the cylinder.

Detection of hamster prion proteins on immunoblots

Samples (15-µl brain homogenates containing native PrP^{Sc}, or PrP 27-30) were subsequently digested with PK (73 µg/ml final concentration in brain homogenates; Sigma, Deisenhofen, Germany) for 1 h at 37ºC. Triplicates of each sample were examined. Positive controls were samples not treated with PK. After denaturation at 95ºC, the samples were separated by 10% polyacrylamide gel electrophoresis. Separated proteins were finally electrotransferred to PVDF membranes (0.2 µm pore size; Bio-Rad Laboratories, Munich, Germany). Surplus binding sites were blocked by incubating the membranes in 5% non-fat dry milk and 5% bovine serum albumin in PBS with 10^{-1}% Triton-X-100. Membranes were then incubated with the anti-hamster PrP^{Sc} 3F4 monoclonal antibody (Signet Laboratories, Dedham, MA, USA) diluted 1:5000 in blocking solution. After incubation with antibodies, membranes were exhaustively washed with PBS/10^{-1}% Triton-X-100 and incubated with peroxidase-labeled goat anti-mouse IgG 1:3000 (Oncogene, Cambridge, MA, USA) in PBS/10^{-1}% Triton-X-100. After further washing, antibody binding was visualized on a highly sensitive Hyperfilm™ ECL™ (Amersham Biosciences, Buckinghamshire, UK) using the enhanced chemiluminescence detection system.

Infectivity bioassays

Infectivity bioassays were performed with the temperature/pressure-treated materials using an incubation time interval protocol (17). After pressurization, ten-fold dilutions (down to 10^{-4}) were intracerebrally inoculated into groups of 4 to 5 weanling hamsters. The highest amount inoculated was 1.5 mg per hamster brain. Untreated or heated samples of the same brain homogenates were examined as controls. Hamsters were observed for clinical signs of scrapie for 270 days and sacrificed immediately after showing disease symptoms. Hamster brains were removed, frozen at -70ºC and assayed after a short period of time in order to detect the PK-resistant form PrP^{Sc}.

Results

Effects of pressure holding time on the proteinase K sensitivity and infectivity of PrP^{Sc}

Samples of brain tissue infected with the 263 K strain of the scrapie agent were exposed to 800 MPa and/or 60ºC for 1 s (decompression immediately after pressure generation) or 5, 30, and 120 min in PBS buffer. After treatment the samples were incubated with (+) or without (-) PK to determine the effect of pressure on the resistance of PrP^{Sc} to proteolytic digestion. Figure 1 shows the immunoblots of treated and untreated PrP^{Sc} detected by the

specific 3F4 antibody. Samples which were only heated at 60ºC for 5, 30, and 120 min were all positive for PrP^{Sc} on the immunoblots and showed no difference compared to untreated controls (results not shown).

Immunoblots of pressurized samples revealed that PrP^{Sc} lost its resistance to proteolytic digestion after treatment, since the typical bands of PrP^{Sc} between 20 and 32 kDa were not detectable on the blot (Figure 1). For the samples pressurized for 1 s and for 5 min a weak but still detectable fraction of PrP^{Sc} remained PK resistant after pressure treatment. If samples were pressurized for 30 and 120 min no PrP-specific proteins could be identified after PK treatment, indicating that the infectious prion protein was efficiently digested by PK up to the detection threshold. The results illustrate that the pressure sensitivity of the prion structure is not immediate and depends greatly on the pressure-holding time.

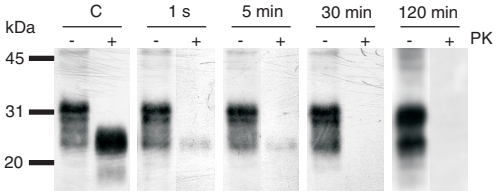

Figure 1. Influence of pressure-holding time on the resistance of PrP^{Sc} to proteinase K (PK). Immunoblot detection of native hamster PrP^{Sc} with the 3F4 antibody. Samples were pressurized in phosphate-buffered saline, pH 7.4, at 800 MPa, 60ºC, for 1 s, 5, 30, and 120 min. Samples were incubated with (+) or without (-) PK. C = control.

Table 1. Incubation period and titer of pressurized samples in bioassay.

	Incubation (days)	Log ID_{50} (u/g brain)
Untreated control	82 ± 8	8.4
60ºC, 30 min	85 ± 5	8.0
60ºC, 120 min	90 ± 6	7.5
800 MPa, 60ºC, 1 s	115 ± 5	5.1
800 MPa, 60ºC, 30 min	134 ± 17	3.5
800 MPa, 60ºC, 120 min	170 ± 38	2.0
700 MPa, 60ºC, 120 min (PK+)	158 ± 23	2.6
900 MPa, 60ºC, 120 min (PK+)	155 ± 26	2.7

Disease transmission to hamster after intracerebral inoculation of 1.5 mg scrapie-infected brain tissue after heating and/or exposure to 800 MPa at 60ºC for 1 s and 30 and 120 min. The 700- and 900-MPa, 60ºC, 120-min samples were digested with proteinase K (PK, 73 µg/ml, 37ºC, 1 h) before *ic* inoculation. Results show the mean incubation time (in days) of animals inoculated with the higher concentration, and the log ID_{50}/(U/g brain). Surviving hamsters were sacrificed after 270 days.

TSE diseases are associated with the accumulation of ß-rich PK-resistant aggregates. This PK resistance is used for the detection of pathological prions. Consequently, if only samples containing PK-resistant prions are considered to be pathological, the homogenates should have lost potential infectivity. To test this, bioassays were performed in hamsters by intracerebral inoculation of the pressure-treated homogenates. Clinical signs of scrapie usually become detectable between 70 and 75 days after inoculation, and death occurs after about 80 days. Animals in this experiment were observed for up to 270 days. Untreated brain homogenates and those treated at 60ºC without pressure for 30 and 120 min led to terminal transmissible spongiform encephalopathy disease after 81 to 89 days (log ID_{50} = 8.4 u/g brain). But the typical incubation times were considerably increased after inoculation of pressure-treated homogenates. The incubation time of the pressurized samples at 800 MPa and 60ºC increased up to 105 to 121 days (log ID_{50} = 5.1 u/g brain) in the case of 1-s treatment, up to 117 to 167 days (log ID_{50} = 3.5 u/g brain) for 30-min treatment and even up to 117 to 210 days (log ID_{50} = 2.0 u/g brain) after 120-min treatment (Table 1). This approximately 10^4 to 10^7 reduction in the ID_{50} units per gram brain in homogenates treated for 30 and 120 min agrees satisfactorily with the decrease in the signal in the immunoblots reported above. All analyzed samples of terminally diseased animals at the end of the reported bioassay were positive for scrapie, independently of the applied treatment (data not shown).

The results indicate that the inactivation of infectious prions by 120-min treatment at 800 MPa and 60ºC could provide an acceptable reduction of infectious units, since oral infection is 10^9 times less effective than intracerebral inoculation (18).

Effects of pH and buffer concentration on the proteinase K sensitivity of pressurized PrP^{Sc}

Figure 2 shows the results after pressurization of PrP^{Sc}-containing brain homogenates at 800 MPa and 60ºC for 120 min in PBS of different pH. In agreement with previous results (19), native infectious prions were found to be pressure sensitive under initially neutral conditions, since no PK-resistant PrP^{Sc} was detectable by specific antibodies after proteolytic digestion. Similar results were obtained when homogenates were pressurized under slightly alkaline pH conditions. Opposite results appeared on immunoblots of samples pressurized at slightly acidic pH, as already reported for the case of acetate (19), where an extensive fraction of PrP^{Sc} remained PK resistant after pressurization in PBS, pH 5.6, and the PK-resistant part of the prion protein was detected (Figure 2).

Figure 3 demonstrates the effect of ionic strength on the PK resistance of pressurized PrP^{Sc}. The samples were homogenized in PBS with different concentrations of salt and phosphate and then treated at 800 MPa and 60ºC for 120 min. Samples pressurized in the less concentrated buffers (Figure 3, samples in 0.1- to 0.5-fold concentrated PBS) showed a weak fraction of PrP^{Sc} remaining PK resistant after treatment. But no PK-resistant prions were detectable on the immunoblots from the samples pressurized in buffers under nearly physiological conditions (Figure 3, samples in 1- and 2-fold concentrated PBS). Samples pressurized in 10-fold-concentrated PBS remained remarkably PK resistant after pressurization, indicating that prions are stabilized under these conditions, an effect comparable to results obtained for samples pressurized at acidic pH (Figure 2).

Discussion

Prions are some of the most resistant pathological agents, surviving all standard inactivation processes such as conventional autoclaving. High pressures at mild temperatures could be a suitable method to inactivate infectious prions. Possibilities of high pressure for the inactivation of pathogenic microorganisms have been intensively explored (20). Only combinations of heat and extreme high pressures are able to inactivate bacterial spores, which are found to be the most persistent species. In a recent paper (16) we reported a decrease in PK resistance of pressurized infectious prions and related to this an extended incubation time of scrapie in hamsters. In an extended study (19), we reported a different pressure behavior of native and isolated infectious prions, demonstrating that only the conformation of native prions is sensitive to pressure.

At present, we have analyzed the influence of the pressure-holding time and pH conditions and also buffer concentration on pressure induced changes in the conforma-

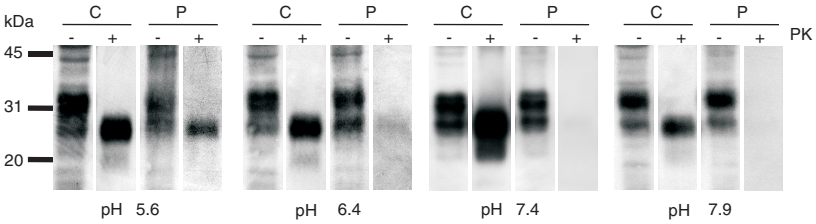

Figure 2. Influence of pH on the resistance of PrP^{Sc} to proteinase K (PK) after pressure treatment. Immunoblot detection of native hamster PrP^{Sc} with the antibody 3F4. C = control; P = treated at 800 MPa, 60ºC, for 120 min in phosphate-buffered saline, pH 5.6, 6.4, 7.4, and 7.9. Samples were incubated with (+) or without (-) PK.

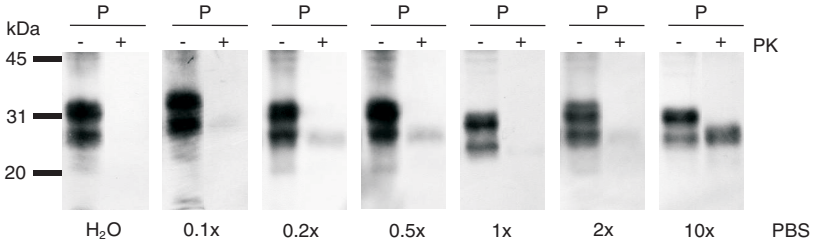

Figure 3. Influence of ionic strength on the resistance of PrP^{Sc} to proteinase K (PK) after pressure treatment. Immunoblot detection of native hamster PrP^{Sc} with the 3F4 antibody. P = treated at 800 MPa, 60ºC, for 120 min in twice-distilled water (H_2O) or in phosphate-buffered saline of different concentrations (0.1- to 10-fold concentrated). Samples were incubated with (+) or without (-) PK.

tion of infectious prions leading to a reduced PK resistance and reduced infectivity. The remarkable phenomenon found in homogenates at initially neutral pH, possibly due to pressure induced changes in the PK-resistant core of prion proteins, is not easy to explain. However, several studies, as the one by Safar et al. (21), have proved that the loss of infectivity quantitatively correlates with a decreasing proportion of native, ß-pleated sheet-like secondary structure components and an increasing amount of α-helical components. Therefore, conditions reducing the content of ß-sheet structure are accompanied by a reduction of infectivity. This could explain our findings that PrP^{Sc} is more PK sensitive and less infectious after treatment with high pressures.

Here we have additionally shown the pressure instability of native prion proteins using a kinetic approach. Pressure is immediately effective already during the slow pressure build-up (up to 120 s to achieve 800 MPa) and most of the detectable PrP^{Sc} is affected by pressure resulting in less PK-resistant detectable materials (Figure 1, 1-s sample). Furthermore, after at least 30 min of treatment with 800 MPa no PK-resistant prions could be detected on the immunoblots. However, the detection threshold of immunoblots, based on the PK digestion of PrP^{Sc}, is rather low. Hence, bioassays give more precise information about the real pressure inactivation effects on PrP^{Sc}. Thus, even though no PrP^{Sc} is detectable on the immunoblots (Figure 1, 30- and 120-min samples), the samples show a relatively high infectivity in the bioassay (Table 1), indicating that a minor fraction of PrP^{Sc} was not affected by high pressure and remained possibly PK resistant although not detectable, and consequently infectious after treatment. We have also digested samples pressurized at 700 and 900 MPa at 60ºC for 120 min with PK (same conditions as used for immunoblot detection) before intracerebral inoculation. Although the PK-digested pressurized samples were PrP^{Sc} negative on the immunoblots (results not shown), they revealed remaining infectivity in the bioassays (Table 1), probably due to a low pressure and thus a PK-resistant fraction of PrP^{Sc}, which is not detectable on immunoblots with specific antibodies.

Bioassay results show that the longer the treatment at 800 MPa and 60ºC the better is the inactivation of infectious materials achieved (Table 1) since the most effective inactivation was observed after the 120-min treatment.

When the scrapie agent is inactivated by autoclaving at 133ºC, destruction of the agent over time follows an exponential function (22). Kinetic studies about scrapie reported by Rohwer (23) have demonstrated that scrapie's resistance to many inactivants is limited to small subpopulations of the total infectivity, the major population being sensitive to inactivation. Our findings are in agreement with the conclusion reached by Rohwer, since even after pressure treatment for 120 min small fractions of the treated scrapie agent remained infectious. Another study by Taylor (24) demonstrated that after autoclaving at 134-138ºC for 18 min the amount of bovine spongiform encephalopathy or scrapie infectivity that survived was relatively constant regardless of starting titer, or whether the agent was in bovine, hamster or mouse brain. The inactivation curve of the 263 K strain of the scrapie agent by autoclaving shows a tailing. Tailing inactivation curves are not uncommon for conventional microorganisms. They may result from protection through clumping, or be due to population heterogeneity where differing straight-line inactivation curves for two subpopulations combine to produce a tailing curve. An explanation for the presence of heat- or chemical-resistant subpopulations of scrapie agents might be the protective effect of aggregation (25). However, aggregates are normally stabilized by hydrophobic or electrostatic interactions at the level of

the quaternary structure, and indeed pressure is usually able to modify the quaternary structure of proteins provoking disaggregation (26,27). Therefore, from our point of view, aggregation is not a convincing explanation for the high resistance of certain prion fractions, and, more likely, the presence of other stabilizing effects, like chaperones (28), or certain characteristics of the secondary structure related to a high content of ß-sheets might be responsible for the high resistance. The resistance of the subpopulations of PrP^{Sc} to heat is difficult to explain by any hypothesis, and in the case of the "protein only" prion model, it implies reversibility of the protein thermal unfolding or a very unusual conformational heat resistance. For example, the loss of free water in the solid state apparently increased the ß-sheet content in the hamster PrP 27-30, leading to higher aggregation and higher thermal resistance (21).

Inactivation of the 263 K strain of scrapie by autoclaving at 134ºC led to a loss of more than seven logs of infectivity, but two logs of the scrapie agent survived the procedure over 1 h (5). High pressure treatment at 800 MPa and 60ºC for 120 min resulted in a similar titer reduction of 6/7 logs of ID_{50} units per gram brain, and also two logs of infectivity remained. The pressure inactivation kinetics of prions can be described by a first order decay reaction as also stated for heat inactivation. The rate of decay obtained is comparable to that induced by autoclaving (data not shown). Remarkable is the fact that the inactivation under pressure starts expeditiously. Indeed, during the pressure build-up (120 s at 60ºC) a notable reduction of 3.3 log occurs, while an equal reduction by autoclaving or by exposure to NaOH takes several minutes. Treatment of infectious materials by high pressure leads to an irreversible and acceptable inactivation, since an oral infection is much less effective than intracerebral inoculation (18). For a feasible industrial application the procedure described here would have to be further optimized.

pH and buffer concentration have been shown to be important parameters in the pressure inactivation of scrapie agents. Results show that initially neutral pH conditions are required for the pressure-induced PK sensitivity of infectious PrP^{Sc}. Initial neutrality of brain homogenates was sufficient for pressure-induced changes in the protein structure even in non-pressure stable buffers such as phosphate buffers (20). Buffers like PBS are not pressure stable in the sense that during pressurization the pH of these buffers shifts towards the acidic region (2-3 units at 800 MPa for phosphate buffer) (29). Therefore, the effect of pressure on the characteristic of the prion structure leading to PK sensibility should have occurred simultaneously with the effect of pressure on pH.

But scrapie prions pressurized in slightly acidic pH remained PK resistant and it may be assumed that a pH-caused change in the protein structure occurring before pressurization may not be reversed during the pressure treatment holding time. The observed protective effect may be interpreted as a pH-induced aggregation of prion proteins associated with changes in protein structure, as it is already known for treatments of prions with acids at atmospheric pressure (30,31). This pH-induced effect was sufficient to inhibit the effects of pressure and those aggregated states induced by low pH remained PK resistant and therefore they should have also remained infectious. It might be possible that the ß-sheet content of the pH-induced aggregates of PrP^{Sc} was higher than in non-aggregated PrP^{Sc}, and therefore they are more pressure resistant, as already reported: studies with the synthetic peptide homologous to residues 106-126 of human PrP, which was previously found to be protease resistant, amyloidogenic and neurotoxic, showed that the ß-sheet content of PrP 106-126 was much higher in phosphate buffer at pH 5.0 than in the same buffer at pH 7.0 (32).

The observed effect of PBS at low concentration on the PK resistance of infectious prions is similar to the effects observed in samples diluted and pressurized in distilled sterile water (Figure 3). In both cases a remarkable decrease of PK resistance occurred and the bands detected on immunoblots were less intense than those of untreated controls. However, the most satisfactory reduction in PK resistance occurs when prions are pressurized in PBS prepared approximating physiological conditions, since here almost no prions were detectable on the immunoblots. In very highly concentrated PBS, PrP^{Sc} molecules seemed to be much more stable against pressure than in PBS of lower concentration. It seems that the structure of PrP^{Sc} is stabilized in strong ionic buffers probably due to local effects on charged amino acids which result in stabilizing the electrostatic interactions. Indeed, it has been reported that intermolecular ß-sheets can be stabilized by electrostatic interactions (33). Furthermore, it is already known that ionic strength highly influences the solubility of proteins and also of PrP^{Sc} in a way that prevents total inactivation through pressure. The effects of pressure may be expected to vary depending on the aggregation level of PrP^{Sc} and indeed pressure-induced changes at the level of the quaternary structure are often reported to be reversible and therefore only measurable *in situ*. Whether additional factors like the theorized chaperones (28) are also playing a role in the stabilization of native prion proteins and in the effects of pressure on the protein structure must be clarified in further experiments. Our results suggest that the use of high pressure processing as a mild decontaminating technology of risk materials could be feasible, although, of course, it still must be optimized. Pressure effects at 60ºC seem to be rapid but an acidic matrix may not be indicated to obtain a good inactivation rate of scrapie prion proteins. Thus, to achieve an acceptable pressure inactivation of infectious prions the raw material should be homogenized in a buffer at neutral pH, taking into account that also the concentration of the buffer involved will be important for PK resistance and probably for the remaining infectivity as well.

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