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Review

Protein conformation determines the sensibility to high pressure treatment of infectious scrapie prions

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Abstract

Application of high pressure can be used for gentle pasteurizing of food, minimizing undesirable alterations such as vitamin losses and changes in taste and color. In addition, pressure has become a useful tool for investigating structural changes in proteins. Treatments of proteins with high pressure can reveal conformations that are not obtainable by other physical variables like temperature, since pressure favors structural transitions accompanied with smaller volumes. Here, we discuss both the potential use of high pressure to inactivate infectious TSE material and the application of this thermodynamic parameter for the investigation of prion folding. This review summarizes our findings on the effects of pressure on the structure of native infectious scrapie prions in hamster brain homogenates and on the structure of infectious prion rods isolated from diseased hamsters brains. Native prions were found to be pressure sensitive, whereas isolated prions revealed an extreme pressure-resistant structure. The discussion will be focused on the different pressure behavior of these prion isoforms, which points out differences in the protein structure that have not been taken into consideration before.
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1. Introduction

Transmissible Spongiform Encephalopathies (TSEs) are a number of fatal diseases generally associated with the accumulation of an isoform of the non-pathogenic cellular prion protein (PrP^C), designated PrP^{Sc}, in brain. Slowly, evidence accumulates pointing out that the misfolding of the cellular isoform into a beta sheet-rich aggregated pathogenic self-propagating multimer is the main step of infection, supporting Prusiner's *protein only model* [1] as a novel way of disease transmission lacking genetic information. But there are still some doubts about the absence of nucleic acid by PrP^{Sc} conversion and aggregation (reviewed in [2]). Nucleic acid molecules are known to bind to PrP^C and are able to stimulate prion conversion [3] and trigger its aggregation into fibrils [4]. TSEs occur in multiple strains characterized by different properties. In infectious diseases such differences generally result from mutations or polymorphisms in the genetic information of the infectious agent. In the prion hypothesis this phenomenon is ascribed to different abnormal conformations of the PrP^{Sc} protein [5]. But the precise structure of the infectious prion protein is not yet known, also the role of the conformation by propagation and aggregation of the prions during pathogenesis. However, even though detailed studies on the structure of PrP^C have been performed, e.g., with NMR [6] and the amino acid sequence is known to be preserved in both isoforms, many features of prions are intriguing since PrP^{Sc} is known to be insoluble. Furthermore, treatments with heat, salts, denaturants and extreme pH revealed new conformational states of prions, different from native ones. But up to now, when a conformational change leads to a reduction in infectivity, inactivation is irreversible [7,8].

The unconventional agents which cause prion disorders are relatively resistant to a wide variety of inactivation procedures

Abbreviations: PrP^C, cellular isoform of the prion protein; PrP^{Sc}, pathogenic isoform of the prion protein; PrP27-30, N-truncated PrP^{Sc}, proteinase K-resistant and infectious core of PrP^{Sc}; iPrP27–30, isolated and resuspended PrP27–30; PK, Proteinase K

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that have an effect on conventional infectious agents [9–11]. Therefore, directives concerning disposal of TSE risk materials are critical, and only extreme inactivation ways of possibly contaminated materials are allowed. Such procedures are exposure to 1 M sodium hydroxide for 1 h at room temperature, gravity-displacement autoclaving at 132 °C for 1 h at room temperature, or porous-load autoclaving at 134–138 °C for 18–60 min. All these procedures though cannot inactivate the TSE-agents completely [12]. Certain expectations exist on alternative milder processes that could provide new sterilization procedures for at-risk materials and may help reducing economical losses in the rendering industry. High hydrostatic pressure is a mild processing technology with a promising potential in food pasteurization and sterilization [13,14]. If verified, an effective inactivation of TSE agents through high pressure processing could improve the security of certain products, such as food of animal origin, animal feed, cosmetics and drugs, while preserving the quality of the treated products.

In addition, high pressure is a thermodynamic parameter providing useful information about the free-energy landscape of proteins [15,16]. High hydrostatic pressure shifts chemical equilibria and accelerates processes for which the transition state has a smaller volume than the ground state. Therefore, unexpected effects different from temperature or chemical denaturation can be seen under pressure and the dynamic development of folding intermediates can be studied [17]. For example, high pressure might stabilize some folded intermediates, as molten-globule conformations [18] or it might increase the density of normal and off-folded intermediates which might provide with amyloid structures constituted by known or new insoluble aggregates [19]. By doing so, pressure brings a new insight in the structure of amyloids and will probably help with the development of pioneering therapeutics.

Our contribution to the present knowledge on pressure effects on prion proteins includes phenomenological studies using the proteinase K resistance of native (PrP^{Sc}), N-truncated (PK-treated PrP^{Sc}, PrP27–30; for more details on the prion nomenclature see reviews [2,20]) and isolated infectious scrapie prion proteins (iPrP27–30) as a direct analytical measure for the effects of pressure on prion conformation. Furthermore, we continuously contrast the obtained immunoblots data with the remaining infectivity of pressurized samples as a measure for the effective inactivation and aiming at observing possible inconsistency between protein conformation and the inactivation rate. The combined pressure/temperature treatments used for these experiments are always below the known denaturing conditions. Results show that pressures at and above 500 MPa and 60 °C at neutral pH are able to induce a remarkable lose in the proteinase K resistance and infectivity of prions which can be attributed to changes in the structure of the prion protein [21]. However, discrepancies under other physico-chemical conditions [22], and between isolated and native prions [23], point out that pressure effects hardly depend on prion conformation and that fractions exist which are especially high pressure resistant.

2. Pressure effects on infectious scrapie prion proteins

The PK resistance of the 263K strain of scrapie prions was investigated in brain homogenates containing the *full length*-PrP^{Sc} (*native* PrP^{Sc}) and the *N-truncated*-PrP^{Sc} (PrP27–30) under pressure/temperature combinations specified below. Homogenization took place in different buffers and in water. Furthermore, infectious scrapie prions were isolated from diseased animal brains and the obtained prion rods (composed of iPrP27–30) were resuspended in different buffers and in water. After pressurization of infectious samples, prion proteins were assessed on immunoblots for their proteinase K (PK) resistance and in hamster bioassays for their infectivity.

2.1. Combined pressure/temperature effects on native infectious scrapie prions

In a series of experiments testing from 100 to 1000 MPa at 60 °C up to 120 min, we could show [21] that the intensity of pressure influences the PK resistance of prion molecules and up 500 MPa, the characteristic prion bands disappear on the immunoblots (Fig. 1, lane 2). Negative blots were at least able to be associated to a loss of 3–4 log of infectivity. However, pressure alone is not able to reduce the PK resistance and infectivity of scrapie prions since pressure treatments at room temperature showed no effect on the infectivity of prions homogenized in PBS pH 7.4 (unpublished data). This way, the amazing effects of pressure on the structure of infectious prions could be observed only by combined pressure/temperature treatments. But the applied temperature is clearly below customary sterilization parameters.

Despite the low sensitivity of available immunoblots [24,25], our bioassays show a reduction down to 6–7 log of infectious units/g in PBS, pH 7.4 at 800 MPa and 60 °C for 120 min. Therefore, this procedure confirms a remarkable reduction of prion infectivity using pressure combined with mild temperatures (Table 1).

The well-accepted explanation for the untypical behavior of the infectious prion protein is the high content of β-sheets in the secondary structure compared to the cellular prion molecule.

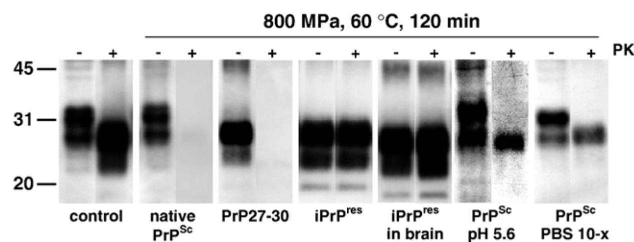

Fig. 1. Effects of pressure on the proteinase K resistance of infectious scrapie prions. Immunoblots of PrP^{Sc} and PrP27–30 in brain homogenates from scrapie diseased hamsters, and of isolated prion rods (iPrP27–30) with the 3F4 antibody. Native PrP^{Sc} and PrP27–30 samples were pressurized in PBS, pH 7.4. PrP^{Sc} were also pressurized in PBS, pH 5.6 and in 10-fold concentrated PBS. Isolated prion rods were pressurized after resuspension in PBS, pH 7.4 and in a healthy brain homogenate (PBS, pH 7.4). Pressure conditions were 800 MPa, 60 °C for 120 min. After pressurization samples were incubated with (+) or without (−) PK. Control sample was untreated material.

Table 1
Proteinase K resistance, incubation period and infectious titer of pressurized infectious native scrapie prions

Samples	PK resistance	Incubation (days)	Log ID_{50} (u/g brain)
Untreated control	resistant	82±8	8.6±0,4
60 °C, 120 min	resistant	90±6	7.3
200 MPa, 60 °C, 120 min	resistant	79±2	8.8
500 MPa, 60 °C, 120 min	sensitive	153±12	2.8
800 MPa, 60 °C, 120 min	sensitive	170±38	2.1±0.2
1000 MPa, 60 °C, 120 min	sensitive	189±39	1.7±0.4
60 °C, 30 min	resistant	85±5	7.7±0.6
800 MPa, 60 °C, 30 min	sensitive	134±17	3.4±0.7

The proteinase K resistance was detected by immunoblots after treatment with PK (73 μg/ml final concentration) for 1 h at 37 °C. Results of the bioassays show the mean incubation time (in days±S.D.) of animals inoculated with 1.5 mg of scrapie infected brain tissue after heating and/or pressurizing at 60 °C for 120 min. Infectivity titer (in log ID50 units per g brain tissue) was calculated by the incubation time interval assay from Prusiner et al. [52].

This probably causes the abnormal resistance of PrP^{Sc} against proteolytic digestion and the consequent formation of high aggregated amyloid-like plaques. Indeed, it has been confirmed that the loss of infectivity of prions quantitatively correlates well with a decreasing proportion of native β-pleated sheet-like secondary structure components and an increasing amount of α-helical components [8]. And moreover, similar effects have been reported by Riesner et al., where SDS caused the disruption of prion rods, generating particles containing α-helices lacking infectivity [26]. One feasible explanation for the loss of infectivity observed in the pressurized samples could be an effect on the conformation of the native PrP^{Sc} molecule at the level of the secondary structure. For instance, only very high pressures (1200 MPa) are able to cause the dissociation of highly aggregated proteins with high β-sheet content under disorganization of the ordered β-structure into α-helical and unordered structures [27]. And in general, that assumption contradicts the known extreme pressure resistance of secondary structures with a high content of β-sheets [27–29].

Other theories, such as the loss of infectivity based on more easily explainable pressure effects on the quaternary structure cannot be confirmed following literature data, since a change at the level of the secondary structure seems to be required. In fact, low pressure levels are able to induce a destabilization of hydrophobic interactions [30], but these effects are usually reversible [31] and would probably not explain the irreversible effect on proteinase K resistance observed under certain pressure/temperature combinations. Unfortunately, the intriguing nature of infectious prions remains a puzzle difficult to elucidate due to the molecule low concentration at the target tissues and the difficulties to isolate the native prion and observe the amyloid plaques without constructing artifacts.

2.2. Combined pressure/temperature effects on the N-truncated-scrapie prions

The *N-truncated* PrP^{Sc} (PrP27–30) showed a similar pressure sensibility in homogenized hamster brains (PBS, pH 7.4). After pressure treatments at 800 MPa and 60 °C for 120 min no PK-resistant protein band could be identified on immunoblots. But the lanes containing no further digested prions (without PK treatment after pressurization) showed a clear positive signal for the prion, confirming that the structural changes induced in the protein have not masked our previous findings. Namely, the antibody was binding to the pressurized protein, and furthermore, native prions were not further aggregated after pressure, since they were effectively entering the gel (see Fig. 1, lane 3). However, the same protein was not detectable after proteinase K digestion showing that certain features of native prion proteins, namely the basic characteristics of the β-rich aggregated pathogenic and PK-resistant multimer were not preserved after pressurization at neutral conditions. Consequently the results obtained by pressurizing PrP27–30 in brain homogenates confirmed the above-described extraordinary behavior of native prion proteins because of the unexpected strong effect of pressure on the otherwise intensely stabilized prion structure.

2.3. Combined pressure/temperature effects on isolated infectious prions

In contrast to the results commented above, isolated and resuspended prions (iPrP27–30) remained PK resistant after pressurizing in several buffers (PBS, pH 7.4; Tris/HCl, pH 7.0) and in water, showing that the conformation of iPrP27–30 was not affected at the same level by pressures up to 800 MPa and 60 °C [23]. It seems that pressure treatments combined with heat at non-denaturing conditions are not able to destabilize the β-structures in isolated scrapie prions (Fig. 1, lane 4) leading to a lower PK resistance.

The same features occurred when purified prions were pressurized after resuspension in brains from non-scrapie hamsters (Fig. 1, lane 5), confirming that no brain-intrinsic factor was responsible for the pressure effects, but probably just the protein conformation.

Further studies during purification of PrP27–30 showed that centrifugation at 540,000×*g* is the critical step causing the pressure resistance of infectious prions (native or N-truncated). It is known that during centrifugation at 540,000×*g* under other conditions, hydrophobic proteins like prions may agglomerate into a highly aggregated hydrophobic core in which proteins are not hydrated. In that cases it might be possible that purification of PrP27–30 induced a semi-crystalline structure which is much more pressure resistant than the native structure. If we consider that protein cavities play an important role in protein denaturation under pressure [32], the structure created during prion purification should have less internal voids than the native hydrated structure and therefore is more stable during pressurization, as it is already known for amyloid fibrils formed from the transthyretin 105–115 peptide [33]. Early aggregated fibrils of this peptide were pressure sensitive, whereas mature fibrils were pressure resistant. It seems that PrP^{Sc} adopts a structure different from native very soon during purification and this could be proven on the basis of the known pressure resistance of certain protein conformations. The purified prions

are still infectious but would behave differently from the native form and would probably have a distinct structure. This would mean that available models on prion structure, which are all based on analysis of isolated PrP27–30 [34,35], and spectroscopic analysis of infectious prions, which were processed by an ultra-centrifugation [36,37], could fail when trying to represent the real features of the native prion since they have been constructed on the bases of a non-native protein structure.

2.4. Effects of pH and ionic strength on the pressure sensitivity of native scrapie prions

The dissociation of certain buffers, as PBS buffer, and water is accelerated by high pressure since equilibrium constants are pressure dependent. Thus, a shift in the acidic region of the pH value of water and the PBS buffer could be expected during pressurization [38]. However, evidence shows that pressure effects on the prion molecule must be immediate during pressurization, since the same pattern of behavior for native prion proteins were able to be observed in PBS buffer or in water, as in TRIS buffer pH 7.0, known to be pressure stable. It seems that a matrix at initial neutral pH is necessary to achieve an effective prion inactivation since all the cases at pH around 7 showed a reduction in the PK-resistant bands after pressurization (Fig. 1, lane 6; for a complete description, see [22]). On the contrary, when initial conditions were set at slightly acidic pH (PBS, pH 5.6, and acetate buffer, pH 5.6), the PrP^{Sc} PK-resistant core was detected on immunoblots after pressurization. Prions aggregate at acidic conditions [39,40] involving a change in the protein structure that could not be reverted during pressurization. Several authors report that prion aggregation at acidic pH could be related to an increase in the amount of β-sheets [41], which would explain the higher pressure stability found in scrapie prions compared to conditions closer to physiological.

Further studies with differently concentrated PBS buffers demonstrated a remarkable influence of salt concentration on the pressure sensitivity of native prions. Similar to the effects found in acidic pH the structure of the infectious native prion protein seemed to be stabilized in 10-fold-concentrated PBS since a remarkable fraction of PK-resistant proteins is detectable on immunoblots (Fig. 1, lane 7; follow complete results in [22]). It is already known that ionic strength highly influences the solution of proteins and furthermore, electrostatic interactions can stabilize intermolecular β-sheets [42]. Hence, the stabilization of the PrP^{Sc} structure in strong ionic buffers is probably due to local effects on charged amino acids which result in stabilizing the electrostatic interactions in a way that prevents inactivation through pressure.

3. Effects on infectivity of scrapie prions of combined pressure/temperature treatments

Up to now, data on pressure effects on prion proteins focus in the non-infectious recombinant prion forms [43]. Slowly, data are recorded that prove pressure to be useful in getting new prion conformations that give an idea of new energy landscapes, but unluckily none of the forms obtained after pressurization have proven to be infectious. In a first approximation to the understanding of pressure effects on infectious prions, one paper dealt with the inactivation of prion proteins using a high pressure assisted sterilization [44]. The temperature shifted up to 135–142 °C within seconds and the authors obtained inactivation rates similar to autoclaving in a processes that could not be directly attributed to pressure, but to temperature or combination of both.

Our studies showed a clear effect of high pressure on the infectivity of infectious scrapie prions at conditions resembling physiological, thus in structures that are considered the more similar to the native molecule. Repeated bioassays with samples that are pressurized at temperatures (60–80 °C) lower than typical denaturation conditions (over 135 °C), showed a reduction in infectivity down to 7 logs ID_{50} u/g if pressure is applied at 800 MPa for 2 h. A 30-min treatment under the same conditions reduced the infectivity up to 5 logs (see Table 1). The pressure inactivation rate at 800 MPa and 60 °C for 120 min is comparable with that obtained by the recommended inactivation procedure for infectious TSE-materials, namely autoclaving at 134 °C for 18 min, which reduces infectivity about 7 logs, while 2 logs of the scrapie agent were infectious after the procedure [45].

The discrepancy about apparently negative immunoblots and positive bioassays illustrates the known low detection threshold of immunoblots for prion proteins. We estimate that a decrease of infectivity below 5 logs brings permanent negative results during electrophoresis in the system we are using, in a way that illustrate the necessity of carrying out bioassays to obtain accurate and sensitive data.

Studies about scrapie inactivation have demonstrated that the resistance of scrapie to many inactivation procedures is limited to small fractions of the total infectivity, while the major conformations being sensitive to inactivation [46]. For example, scrapie inactivation by autoclaving at 133 °C shows a destruction of the agent over time which follows an exponential function [10,47] comparable to the results with pressure combined with heat below denaturing conditions (Fig. 2). Although inactivation with high pressure was impressive and irreversible, also a remaining active fraction was still detectable

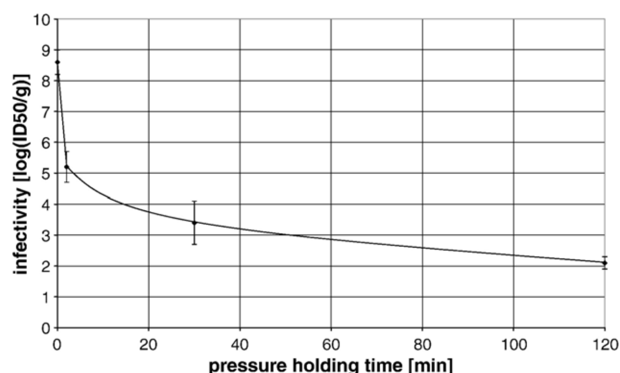

Fig. 2. Inactivation curve of the 263 K strain of scrapie during pressurizing at 800 MPa and 60 °C.

after 2-h treatment, resembling other inactivation methods. The pressure inactivation kinetics can be described by a first order reaction as stated for heat inactivation before. Since an oral infection with prions is 10^9 less effective than intracerebral inoculation [48], high pressure treatment of infectious materials might then lead to an acceptable inactivation if treatments are prolonged enough.

The protective effect of special protein conformations and subsequent prion aggregation is a feasible explanation for the presence of heat- or chemical-resistant fractions [49]. Here, we have reported that the usually most heat-resistant protein conformations are also the most pressure stable, confirming that prion hydration plays an important role also to inactivate infectious prions [50,51].

4. Concluding remarks

The pathogenic scrapie prion protein 263K in its native form at initial neutral conditions proved to be highly pressure sensitive. We could confirm after having done several experimental tests that pressure combined with heat below the known denaturing conditions was able to induce changes in the protein conformation which resulted in the loss of PK resistance and infectivity. Similar results were obtained by pressurizing the *N*-truncated prion protein, PrP27–30, in brain homogenates which showed also a remarkable loss of PK resistance after pressure treatment. In contrast, isolated prion proteins (iPrP27–30) demonstrated a extraordinary stability to high pressure treatments since pressure was not able to induce changes in the structure of prions after centrifugation at 540,000×*g* and the PK-resistant fraction remained detectable after treatment. Likewise, the native scrapie prion (PrP^{Sc}) showed a similar high pressure stability when pressurized at acidic pH and in highly concentrated buffers.

In general, our results confirm that the biggest amount of prions in conditions close to native are pressure sensitive and loose infectivity soon during pressurization, but pressure-resistant coexisting fractions cannot be discarded. Furthermore, any treatment leading to more aggregated and dehydrated structures result in an increased resistance to high-pressure treatments. Thus, the inactivation of infectious prions seems to be highly dependent on protein conformation. As a result, we must accept that a certain conformation has pressure-sensitive β-structures (native prions) and another conformation has pressure stable β-structures (stronger aggregated prions less hydrated and with smaller cavities), and they could coexist. The stabilization of the native structure through the formation of differently ordered beta-sheets would then be crucial for the pressure stability of infectious prions. This suggests that the extreme robust prion aggregates are not stabilized by the quaternary structure, usually sensitive to pressure, but rather through newly ordered β-sheets with less voids, which are supposed to be extremely pressure stable. This explanation assumes the knowledge coming from the "protein only" theory and the accepted concepts of protein behavior under pressure and is difficult to prove because of the limited accessibility of native infectious prions, but it allows us to think that actual models on prion structure might be based in highly ordered structures not equal to less ordered native prions.

Up to now, our results prove that pressure (over 800 MPa) combined with medium heat (not over 60 °C) are enough to cause an irreversible and acceptable inactivation of certain fractions of infectious prions. By optimizing the pressure conditions, mild inactivation of contaminated materials are possible with this technology. Furthermore, pressure combination with other denaturants different to heat might let us obtain new insights into the prion behavior getting new parameters to clarify the real impact of pressure on protein structure. Much work remains to be done.

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