Prion depletion and preservation of biological activity by preparative chaotrope ultracentrifugation

Seeger, H; Julius, C; Cozzari, C; Calella, A M; Dattilo, M; Aguzzi, A
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Abstract

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Prion depletion and preservation of biological activity by preparative chaotrope ultracentrifugation

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Abstract

Prions are characterized by unusual physicochemical properties, such as insolubility and resistance to proteases, and maintain infectivity after contact with disinfectants and decontamination procedures active against conventional pathogens. To date, most methods for prion inactivation are either incomplete or unacceptably harsh for the purification of fragile biotherapeutics. Here we describe a simple prion removal procedure that takes advantage of differential sedimentation and denaturation of prions. Prion-spiked fluids were layered onto an intermediate sucrose cushion and an 8M urea solution, and subjected to single-step ultracentrifugation. Due to their insolubility, prions rapidly traveled through the sucrose cushion into the urea solution. Prion infectivity in the upper phase was reduced by at least 3.2 logs, or up to 6 logs or more. Very little soluble protein was lost from the input sample and a proof-of-principle experiment demonstrated only marginally reduced biological activity of spiked enzyme after ultracentrifugation. This procedure is likely to synergize with nanofiltration and other prion removal steps in the treatment of batches of raw and semifinal biopharmaceutical materials.
Introduction

Prions are infectious agents causing invariably fatal neurodegenerative diseases, such as Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep and goats [1, 2]. Prions are characterized by certain unusual physicochemical properties, and, in particular, by their strong resistance to common procedures of decontamination used against conventional microorganisms [3]. According to the protein-only hypothesis, PrP^{Sc}, and abnormally folded and aggregated isoform of the host-encoded cellular prion protein PrP^{C}, constitutes the major component of the prion [4].

Efficient physicochemical decontamination methods for most strains of prions include exposure to 1 M sodium hydroxide for one hour at room temperature, porous-load autoclaving at ≥134 °C for at least 60 min, or gravity-displacement autoclaving at 132 °C for one hour [5]. Sodium hypochlorite solutions containing 20'000 ppm of available chlorine have also been reported to be effective [6, 7]. Furthermore, combining exposure to sodium hydroxide with autoclaving has been described to completely eliminate prion infectivity [8]. However, biological materials typically do not withstand the harsh or denaturing methods that would guarantee complete inactivation of prions. Hence alternative approaches are required for efficient and substantial reduction of the infectious agent in a milder fashion, such that native properties of the biological to be processed are maintained intact. For the production of therapeutics from blood, plasma or other biological fluids such as urine or tissue extracts, the purified compounds need to be separated from the prions during biomanufacturing processes such that biological activity of the desired therapeutic is assured. Many attempts to remove prions from biological materials have been performed using precipitation, chromatography, and filtration [9-12]. During the manufacturing or isolation processes of therapeutic compounds from biological fluids, it is desirable to combine several of these steps consecutively, since none of them guarantees complete removal of prions.

Non-denaturing prion removal approaches are not only required for the production of therapeutics, but also for decontamination of samples for research purposes. This is particularly important when downstream analytical procedures need to be performed outside
a designated biosafety laboratory. This is often the case when bulky or extremely expensive apparatuses, such as e.g. mass spectrometers, liquid chromatography units, nuclear magnetic resonance spectroscopes, cannot be hosted within biosafety laboratories.

The aim of this study was to establish an alternative approach to the decontamination techniques described above, using milder procedures that may be applicable to delicate biological samples. Towards this goal, we strived to establish a preparative technique that would be suitable for the removal of the infectious agent from biological fluids or tissue extracts containing soluble proteins or other small non-proteinaceous molecules of interest, such as metabolites. Ultracentrifugation was shown to efficiently sediment the infectious prion agent [13, 14]. The principle underlying the procedure described here is that prions contained in biological fluids will sediment during ultracentrifugation. This property is derived from the aggregated nature of PrP\textsuperscript{Sc}, and can be exploited to reduce – or possibly clear – the infectious agent from the solution containing the biological of interest.

We reasoned that using a step gradient with a denaturing bottom fraction would inactivate prions that were sedimented by ultracentrifugation, while leaving compounds in the top fraction biologically active. The primary advantage of the non-denaturing removal procedure described in this study is that specialized equipment, such as nanofiltration or chromatography devices, is not required. Another noteworthy aspect is that this technique avoids precipitation of the sample by organic compounds such as acetone or trichloroacetic acid, which may potentially lead to irreversible inactivation of biologically active proteins, or to undesired coprecipitation of the molecules of interest along with the infectious prions. As with most other “soft” prion removal technology, complete sterilization was not always achieved, yet the reduction factors attained by step gradient ultracentrifugation were sufficient to warrant its use in combination with other technologies.
Materials and Methods

Preparation of RML5 mouse scrapie and mock brain homogenates: Homogenates were prepared from terminally scrapie-sick CD1 mice that had been inoculated with passage 4.1 of the RML mouse prion strain (RML5 infectious inoculum), or from healthy CD1 mice (mock inoculum). Brains were homogenized in 10 volumes of 0.32M Sucrose with a Polytron homogenizer. Homogenates were clarified for 1h at 1’000g, and aliquots of the supernatant were frozen at –80 °C.

Ultracentrifugation: Ultracentrifugation was performed in a Sorvall Discovery M150E centrifuge using a Sorvall S55S swinging bucket rotor (r_{avg} 6.1 cm). Centrifugation was performed at 40’000rpm (RCF=110’000g) for 2 hrs at 4 °C. The top fraction (10mg/ml BSA (Sigma, USA) in PBS) was spiked with 10% of RML5 mouse scrapie brain homogenate (1:10). The intermediate fraction consisted of 10% sucrose (Sigma) containing bromophenol blue (Merck, USA), whereas the bottom fraction was composed of 8 M urea in water (Sigma, USA). Fractions were loaded from top to bottom: 1 ml of the top fraction was carefully pipetted into the ultracentrifuge tube. Using a 1 ml syringe with a long needle 0.5 ml of the intermediate fraction was carefully added to the bottom of the tube, followed by 0.5 ml of the bottom fraction. After centrifugation, fractions where collected from top to bottom (Fig. 1). Firstly, 0.5 ml were removed from the top fraction (T1). This was followed by the second 0.5 ml of the top fraction (T2), the intermediate fraction (I) and the bottom fraction (B).

Sodium phosphotungstate (PTA) precipitation: PTA precipitation was performed similarly to previous reports [15]. PBS (50 μl) was added to 450 μl of each sample, and followed by the addition of an equal volume of 4% Sarkosyl in PBS. The samples were then mixed, and incubated for 15 min at 37°C under constant agitation. Benzonase and MgCl₂ were added to a final concentration of 50 U/ml and 12.75 mM, respectively, and samples were incubated for 30 min at 37°C under continuous agitation. Pre-warmed PTA (pH 7.4) was added to a final concentration of 0.3%, and samples were incubated at 37°C for 30 min with constant
agitation, followed by centrifugation at 37°C for 30 min at maximum speed in a benchtop Eppendorf microcentrifuge. Resultant pellets were resuspended in 20μl PBS containing 0.1% Sarkosyl, and digested with proteinase K (PK; 50 μg/ml) for 1h at 37°C under continuous agitation. Samples were heated at 95°C for 5 min in SDS-containing loading buffer prior to gel loading.

**Western blot analysis:** Samples were electrophoresed through 12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen, USA) at 150V MES running buffer (Invitrogen). Transfer was performed for 2h at 110V in transfer buffer (25mM Tris, ph 8.3, 0.19M glycine, 20% methanol) onto nitrocellulose membranes (Schleicher-Schuell, Germany). Membranes were blocked with TBS-T containing 5% Topblock (Juro, Switzerland), incubated with monoclonal anti-mouse PrP POM1 antibody [16], diluted 1:3000 in TBS-T containing 1% Topblock, at 4°C overnight. For detection, HRP coupled rabbit-anti mouse IgG1 antibody (Zymed, USA) was used at a dilution of 1:10.000 in TBS-T, containing 1% Topblock. Following incubation in Supersignal ECL reagent (Pierce, USA), membranes were exposed to ECL Hyperfilm (Amersham, UK).

**Coomassie blue staining:** Each sample (amounts as indicated) was electrophoresed through 12% NuPAGE gel. Following electrophoresis, gels were transferred to a Coomassie blue bath (0.025% Coomassie Blue R250, 40% Methanol, 7% acetic acid) and incubated for 2 hrs. Gels were subsequently destained in several changes of 40% methanol and 7% acetic acid for several hours.

**Dialysis:** Samples from fractions (I) and (B) were dialyzed prior to use in cell culture experiments, using Slide-A-Lyzer Dialysis Cassettes with 3.5kD molecular weight cut-off, 0.1 - 0.5 ml capacity (Pierce, USA). Dialysis was performed for 2h at room temperature against 2 l of PBS, with two changes of the dialysis buffer.
Scrapie cell assay in endpoint format (SCEPA): Cells were cultured in Optimem (GIBCO, USA) containing 10% fetal calf serum (Hyclone, Perbio, Switzerland), 2mM L-glutamine in the presence of 10,000 U/ml Penicillin and 100μg/ml Streptomycin (Invitrogen, USA). For SCEPA, highly prion-susceptible neuroblastoma cells (subclone N2aPK1 [17]) were exposed to prion-containing samples in 10-fold dilutions for 3 days in 96-well-plates. Cells were split three times 1:3 every two, and three times 1:10 every three days. After reaching confluence, 25’000 cells from each well were filtered onto the membrane of an ELISPOT plate (Millipore), treated with proteinase K (PK), denatured using 3M GdHCl, and individual infected (PrPSc-positive) cells were detected by in situ immunocytochemistry using monoclonal antibody POM1 to PrP in a 1:50 dilution. Wells were scored positive if the spot number, as assessed by visual inspection, exceeded the background in cells incubated with mock homogenate. The prion infectivity titers (Tissue culture infectivity dose: TCID50) were calculated from the numbers of negative and total wells using the statistical treatment of Reed and Muench [18]. The number of “infectious tissue culture units (TCI)” per aliquot was calculated using the Poisson equation. The exquisite sensitivity of the SCEPA in the format used here is based on the fact that the proportion of infected cells, and with it the signal-to-background ratio, increases on average about 25% per day during culturing [17].

Infectivity bioassays with tga20 indicator mice: From each sample 30 µl was inoculated intracerebrally into each of four tga20 mice, which overexpress PrPC and are utilized as sensitive indicators for prion infectivity [19]. Infectivity titers were calculated using the relationship y=11.45-0.088x (y, logLD50 per ml of homogenate; x, number of days from inoculation to terminal disease) which had been derived from titrations of standard inocula as described [20].
**Horseradish-peroxidase (HRP) activity measurement:** The top fraction (10mg/ml BSA (Sigma, USA) in PBS) was spiked with 10% of RML5 mouse scrapie brain homogenate (1:10) and 1μl (1:3000) rabbit-anti-mouse IgG coupled to horseradish-peroxidase (HRP; Zymed/Invitrogen, USA). Intermediate and bottom fraction as well as ultracentrifugation were performed as described. After ultracentrifugation HRP activity was measured by luminometry after addition of a chemiluminescent substrate (SuperSignal ELISA Pico; Pierce, USA) in the input (Ipt) as well as in all fractions after ultracentrifugation. PBS served as a negative control. Two independent experiments were performed where three replicates per fraction were measured.
Results

Step gradient layers remain separated after ultracentrifugation.

We performed zonal ultracentrifugation using a step gradient consisting of three layers of increasing density (Fig. 1). The bottom layer (B) was of high density and contained chaotropes in sufficient concentration for inactivation of prions. In the experiments described here, 8 M urea was used [21] because it is relatively non-toxic and can be utilized in the manufacturing of biologicals, but other chaotropes could also conceivably be used. The bottom layer was overlaid with an intermediate layer (I), which consisted of a solution with density (10% sucrose) intermediate between that of the top and the bottom layers. For the purpose of visualization, the intermediate layer contained bromophenol blue. The top layer (T) had the lowest density and consists of the biological sample to be decontaminated. Importantly, the intermediate layer was utilized to ensure separation of the denaturing bottom layer from the top layer containing the biological of interest.

Ultracentrifugation of the discontinuous gradient for 2h at 110,000g resulted in recovery of three intact fractions with minimal spreading of the top fraction (T) into the intermediate fraction (I), and very little contamination of the intermediate fraction with the bottom fraction (B).

Minimal loss of soluble proteins from top layer.

During the prion reduction procedure we strived to minimize any loss of proteins from the top fraction, as this fraction contained the components to be purified and/or analyzed in subsequent downstream procedures. In order to assess any potential protein losses, aliquots from each fraction were electrophoresed and gels were stained with Coomassie blue to visualize protein composition of each gradient layer. Fig. 2 shows the proteins present in the different fractions of an experiment performed with bovine serum albumin (BSA) in the top layer. Predominant amounts of protein were observed in the top layer (T1, T2) after ultracentrifugation. Only small amounts of protein were found to have migrated or diffused to
the intermediate (I) or bottom fraction (B), whereas the bulk of protein was stationary to the top phase.

Efficient removal of PrP$_{Sc}$ following step gradient ultracentrifugation.

To assess the removal of PrP$_{Sc}$ in the top layer following ultracentrifugation, we performed western blot analyses of samples from each layer (T1, T2, I, B) following ultracentrifugation, and compared them to the top layer prior to ultracentrifugation (Fig. 3A). To distinguish between PrP$_C$ and PrP$_{Sc}$ we digested 20 μl of each fraction with PK where indicated. In two independent experiments, we were unable to detect PrP$_{Sc}$ in aliquots of the top layer (T1, T2), even after overexposure for 3 hr (Fig. 3A). Furthermore, no PrP$_{Sc}$ was detected in the intermediate layer (I). In one experiment we detected traces of PrP$_{Sc}$ in the bottom fraction (B) (Fig. 3A), whereas in a second experiment we were not able to detect PrP$_{Sc}$ in the bottom fraction (data not shown). As expected, large amounts were present in the top fraction (Ipt) before centrifugation. Notably, the bottom fraction (B) exhibited a strong signal for PrP$_C$, comparable to the PrP signal in the top fraction prior to ultracentrifugation (Ipt). To increase the sensitivity of the Western blot assay for PrP$_{Sc}$ we performed a phosphotungstic acid precipitation using a total volume of 450 μl for each fraction. This was found to lead to an approximately twenty-fold enhancement in sensitivity. In two independent experiments we could detect PrP$_{Sc}$ signals in precipitates of the intermediate and the bottom fractions (Fig. 3B). Small amounts were also detected in one of two analyses of the T2 fraction (Fig. 3B left panels) and possible traces were also found in the T1 fraction of the second experiment (Fig. 3B right panels).

Reduction of prion infectivity by step gradient ultracentrifugation.

Since the amount of PrP$_{Sc}$ does not always correlate directly with the level of infectivity [22], we performed a scrapie cell assay of the different fractions after ultracentrifugation to assess the reduction factor in prion infectivity titers. Fractions I and B were dialyzed prior to SCEPA
to remove urea, which would have been toxic to neuroblastoma cells. Dialysis was chosen rather than dilution in order to maintain a maximal sensitivity of the assay and avoid possible false negatives in the post-procedure readout. Importantly, we have shown that the procedure of dialysis does not reduce prion infectivity in a given sample [23]. If anything, nominal infectivity tends to increase after dialysis, possibly because of the removal of small-molecular inhibitory constituents of brain homogenate. Hence, log reduction calculations based on SCEPA after dialysis are most certainly not overoptimistic.

Two independent experiments with the same input infectivity were carried out in order to estimate the reproducibility of the method. As prion spike, we used the mouse-adapted Rocky Mountain Laboratory scrapie strain “RML5”. The titer of this homogenate had been previously ascertained in a mouse bioassay [24]. As shown in Table 1, the prion titer in the top layer prior to ultracentrifugation was approximately 7 log TCI units/ml. After ultracentrifugation, fractions were collected from top to bottom. In two experiments performed on different weeks, there was a reduction of prion infectivity in the top fraction by 6.1 (T1) and 4.8 (T2) in experiment 1 and by 5.2 (T1) and 4.5 (T2) log TCI units/ml in experiment 2. Prion infectivity in the intermediate fraction (I) was 2.2 and 1.6 log TCI units/ml whereas infectivity in the bottom phase (B) reached values of 2.2 and 2.6 log TCI units/ml, respectively.

To verify these results in a second paradigm, we measured prion infectivity in the different fractions after ultracentrifugation by mouse bioassay utilizing tga20 mice, which overexpress Prnp and represent a convenient and relatively fast model for sensitive detection of prions [25]. Dialyzed fractions I and B were inoculated intracerebrally (i.c.) into tga20 indicator mice, which were subsequently monitored for signs of prion disease. Again, the same input was used in two temporally independent experiments, with mouse scrapie brain homogenate RML5 as a prion spike. As shown in Table 2, the prion titer in the top layer prior to ultracentrifugation was approximately 6.6 log LD50 units/ml. After ultracentrifugation, fractions were collected from top to bottom. In two experiments performed there was a reduction of prion infectivity in the top fraction by 4.3 (T1 and T2) in experiment 1 and by 3.6 (T1) and 3.2
Prion infectivity in the intermediate fraction (I) was 3.7 and 4.1 log LD$_{50}$ units/ml whereas infectivity in the bottom phase (B) reached values of 3.0 and 3.6 log LD$_{50}$ units/ml.

The sensitivity of the SCEPA assay, as performed here, was in the same order of magnitude of the mouse bioassay using $tga20$ transgenic mice [19]: the nominal difference in sensitivity of approximately 0.5 logs lies within the standard error of the mouse bioassay (typically ±1 log when performed with 4-6 mice/titration step).

### Preservation of enzyme activity after step gradient ultracentrifugation.

We then went on to investigate whether the procedure presented here would be applicable to prion removal from biological fluids without impairment of compounds to be purified. Towards that goal, we investigated the impact of step gradient ultracentrifugation on a sample spiked with prions and horseradish-peroxidase (HRP; Fig. 4). A commercial HRP-antibody conjugate was used in order to simulate the purification of realistic macromolecules. In agreement with the experiments detailed above, step gradient ultracentrifugation once again resulted in removal of PrP$^{Sc}$ from the top layers (T1 and T2) and no PK resistant PrP was detected in the intermediate (I) and bottom (B) fractions after ultracentrifugation as assessed by Western blot analysis (Fig. 4A). In stark contrast, approx. 70% of the input HRP activity remained in the top fractions. Less than 30% of the input enzyme activity was detected in the intermediate fraction, and only traces of enzyme activity were measured in the bottom fraction (Fig. 4B).
Discussion

Prevention of iatrogenic transmission of infectious agents is crucial to the production of biotherapeutical compounds for human or veterinarian use. Preventive strategies are typically based on three pillars: (1) sourcing of uncontaminated raw materials, (2) sensitive diagnostic tests, and (3) removal and inactivation procedures. This is particularly important in the case of prions, for which no effective therapy exists.

Furthermore, prion decontamination methods are required in biological research in order to avoid occupational exposure to TSE agents, and also to minimize contamination of expensive technical devices. One such example for the latter situation is the search for biomarkers of prion diseases in body fluids such as blood, urine, CSF, and saliva. Such markers would be invaluable for the early diagnosis of prion disease and/or for monitoring therapeutic efficiency of future treatments. However, approaches for the identification of biomarkers often involve the use of expensive equipment such as mass spectrometers or sophisticated chromatography devices, which are rarely not available to laboratories that have the biosafety status needed to handle prion agents and cannot be readily decontaminated with harsh standard prion disinfectants such as sodium hydroxide or hypochlorite [26].

Inactivation of prions by physical and/or chemical procedures is proverbially difficult to achieve [27, 28]. Those methods which are known to reliably inactivate prion agents are far too harsh to be used for direct treatment of biologically fragile active substances derived from serum, plasma, urine or cell culture products. Therefore, the spectrum of available methods for prion load reduction from biologicals is currently very limited. In practical terms, the only procedures that are typically available make use of certain precipitation methods, separation by chromatography, and/or removal by filtration. However, when employed as the only prion reduction strategy, most of these methods rarely guarantee highly efficient removal of prions [10, 12]. Therefore, a commonly used approach consists of using several of these methods sequentially. This approach works best if the chosen methods are “orthogonal”, i.e. they rely on fundamentally different physical principles: the sequential application of two methods...
based on size-exclusion, each one of which may have a prion reduction power of 3 logs, will certainly not amount to 6 logs as the efficacy of the second step will have been preempted by the first step.

Accordingly, there is a great need to invent additional methods of prion decontamination that are based on novel biophysical principles. For these reasons, we sought to develop an additional preparative procedure, which is suitable to deplete prions from biological samples, which could be used in biomedical research and, importantly, for the treatment of materials during the biomanufacturing process of therapeutics intended for use in humans.

The prion removal procedure presented here is based on ultracentrifugation, in which prions contained in homogenates or biological fluids efficiently sediment during high-speed centrifugation, whereas soluble proteins of interest do not readily sediment. The biophysical underpinnings of this procedure rest on the presumption that prions consists obligatorily of protein aggregates [29]. Accordingly, it was reported that centrifugation of clarified 10% mouse scrapie brain or spleen homogenates at $\omega^2t$ values of greater than $1.0 \times 10^{11}$ rad$^2$/s (centrifugation for 1h at 50,000 rpm with Beckman 50Ti rotor $r_{avg}5.9\,\text{cm}$) reduced the prion titer in the supernatant by approximately 4 logs [14]. Ultracentrifugation has the advantage that it allows soluble proteins or other compounds to remain in solution, whereas non-soluble or aggregated compounds such as prions will sediment.

Previous studies of prion removal have typically made use of either brain homogenate or of microsomal fractions as spikes [30]. Since the actual biophysical structure of prion infectivity in blood or urine remains elusive, we opted for utilizing prion infectivity derived from brains of infected animals as an appropriate source of infectivity for this kind of studies. In agreement with this estimation, recent publication have concluded that there is no proof that any brain-derived material is suitable or unsuitable for spiking [31]. For ultracentrifugation we used a step gradient as described in Fig. 1. As such, reduction of prion infectivity in the biological sample can be achieved by sedimentation and at the same time inactivation of sedimented prions occurs at the bottom of the tube. To avoid mixing of the urea fraction with the sample
used to derive biotherapeutics, we included an intermediate layer (10% sucrose) which physically separates the sample layer and the inactivation layer, but which can still be penetrated by prions. We chose urea as an inactivation solution since it has been established that incubation of prion containing preparations in 8 M urea leads to a reduction of prion infectivity by 5-6 logs [21]. Additionally, urea is a significantly milder denaturant and much less toxic than guanidinium thiocyanate or guanidium chloride. Sodium hydroxide is also a highly effective denaturant that is capable of sterilizing prions, but the chemical hazards associated with the handling of large amounts of sodium hydroxide rendered this decontaminant less attractive.

By utilizing a step gradient ultracentrifugation procedure, we have achieved reduction of prion infectivity in the experimental samples by a factor of at least 3.2 logs. In some experiments, a reduction factor of >6 logs was found. Moreover, we found that only minimal soluble protein was lost from the input sample. Most importantly, bioactive compounds such as HRP were found to retain their biological activity after centrifugation and subsequent recovery from the top fraction.

In the Western blot depicted in Fig. 3A, the signal for total PrP in the bottom fraction after centrifugation was similar to the signal for total PrP in the top fraction before centrifugation. This indicates efficient sedimentation of PrP. Despite similar amounts of PrP in the top phase before and the bottom phase after centrifugation, only relatively little PrP$_{Sc}$ was detected in the bottom fraction compared to the large amount in the input. The finding that almost no PrP$_{Sc}$ was detected by PTA precipitation in the top and only little in the intermediate fraction after centrifugation suggests that the majority of PrP$_{Sc}$ had sedimented to the bottom phase and was rendered proteinase K sensitive by the urea in this fraction. Loss of PK resistance of PrP$_{Sc}$ is usually accompanied by a reduction in prion infectivity [32]. This was reflected by the low infectivity titer in the bottom phase (2.2 - 2.6 log TCI units/ml or 3.0 - 4.1 log LD$_{50}$ units/ml), when compared to the top phase before centrifugation (6.7 log TCI units/ml or 6.6 log LD$_{50}$ units/ml). If inefficient inactivation of prions had taken place, the infectivity in the bottom fraction would have been similar to that contained in the top fraction prior to
ultracentrifugation. The 0.5 logs lower sensitivity of the SCEPA compared to the MBA in our study is most likely caused by biological variation of the susceptibility of the N2a subclone used [33].

Whilst absolute removal of prions was not achieved using the preparative procedure outlined here, our observed reduction of 3.2 - 6 logs represents a substantial minimization of prion infectivity and is similar, or in some cases superior, in its efficiency to other non-denaturing approaches reported by others. Certain nanofiltration procedures for example have accomplished reduction in prion infectivity between 1.6 and 5.9 logs, depending on the nature of the prion spike and the filters used [12, 34-36]. However, one problem with filtration is that filters tend to foul if tissue homogenates, solutions of high protein content or certain detergents are used [11]. In a well-performed study, ion exchange chromatography has been shown to decrease prion infectivity by 4-5 logs [10]. Whereas filtration or chromatography systems are routinely used by companies for the reduction of viruses in large scale manufacturing processes, they require specialized equipment, which is often not available in typical research laboratories. Precipitation has also been demonstrated to be useful for prion decontamination during manufacturing processes and reduction factors of 1–6 log LD50 units have been reported using methanol or ethanol in combination with acidic pH [10, 37, 38]. However, precipitation has the disadvantage that potential therapeutic proteins of interest might co-precipitate with prions (which is also disadvantageous for proteomics research studies) or that precipitated proteins may become inactivated by treatment with organic solvents.

The method we have established might be used in prion research as prion depletion procedure for samples which contain low amounts of prions such as blood, urine or cerebrospinal fluid [39-41] or for fluids where infectivity has not yet been convincingly demonstrated, but which potentially contain low amounts of prions. If employed for samples containing high amounts of prions such as brain or spleen homogenates it should be used in conjunction with a second method. The method described here can also be applied to the isolation of therapeutics from biological samples of human or bovine origin. Importantly,
centrifugation is truly “orthogonal” to filtration procedures, and it can be very easily combined with e.g. nanofiltration in order to attain maximal prion load reduction. A further conclusion that can be drawn from this study is that the western blot assay for PrP$^{\text{Sc}}$ alone is not sufficient for the validation of methods aiming at prion decontamination. We have demonstrated by using the PTA precipitation that the sensitivity of the Western blot assay for validation studies can be significantly increased. However, measuring the prion infectivity in a sample by bioassay remains the gold standard for such approaches. For validation studies where mouse prions are used as a prion spike, the scrapie cell assay in endpoint format represents a cost efficient and less time-consuming alternative to the mouse bioassay.

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References


Figure legends and tables

Figure 1: Preparative step gradient ultracentrifugation procedure.
The upper fraction (T) consists of the biological sample to be decontaminated. It is separated by a fraction of intermediate density (I) from the denaturing bottom fraction (B). During ultracentrifugation the infectious prions will sediment to the bottom of the tube where they become inactivated.

Figure 2: Distribution of proteins in step gradient layers before and after ultracentrifugation.
Proteins from fractions T1-B after ultracentrifugation compared to proteins in the input (Ipt). Note the minimal loss of protein from the top fraction into the intermediate (I) or bottom (B) fractions.

Figure 3: Efficient reduction of PrP<sup>Sc</sup> by step gradient ultracentrifugation.
A) Western blot of 20µl of each fraction T1-B after ultracentrifugation. After ultracentrifugation PrP<sup>Sc</sup> cannot be detected anymore in the top fractions T1 and T2. Ipt = top fraction before ultracentrifugation. Minimal amounts of PrP<sup>Sc</sup> could be detected after centrifugation in the bottom phase (B) in the experiment presented here (long exposure). Note the strong signal for PrP in the bottom phase after centrifugation.
B) Western blot after PTA precipitation of 450µl of each fraction T1-B after ultracentrifugation. Two representative experiments are shown. For the input sample only 10µl were used. After PTA precipitation signals for PrP<sup>Sc</sup> could be detected in the intermediate (I) and bottom (B) fractions of both experiments, whereas only minimal amounts were detected in fractions T1 and T2.
Figure 4: Preservation of enzyme activity after step gradient ultracentrifugation.

A) Western blot of 20μl of each fraction T1-B after ultracentrifugation. After ultracentrifugation PrPSc can not be detected anymore in the top fractions T1 and T2. Ipt = top fraction spiked with prions and horseradish-peroxidase (HRP) coupled to antibodies before ultracentrifugation.

B) Enzyme activity of each fraction T1-B after ultracentrifugation. Horseradish-peroxidase (HRP) activity was normalized to Ipt (input sample was spiked with prions and HRP coupled to antibodies) whereas PBS served as negative control.
Table 1: Prion infectivity in the different fractions after step gradient ultracentrifugation measured by SCEPA.

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<th>Fraction</th>
<th>Experiment 1 log Reduction †</th>
<th>Experiment 2 log Reduction †</th>
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<tr>
<td>Input‡</td>
<td>6.7 (7.0)¹</td>
<td>6.7 (7.0)</td>
</tr>
<tr>
<td>T1</td>
<td>0.6 (&lt;1.5)</td>
<td>6.1 (&gt;5.5)</td>
</tr>
<tr>
<td>T2</td>
<td>1.9 (2.1)</td>
<td>4.8 (4.9)</td>
</tr>
<tr>
<td>I</td>
<td>2.2 (2.3)</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>2.2 (2.4)</td>
<td>2.6 (3.1)</td>
</tr>
</tbody>
</table>

*One tissue culture infectivity (TCI) unit is the amount of infectivity needed to infect one well (=one infectious particle).

‡Top fraction before ultracentrifugation
† Reduction of prion infectivity titer in log TCI units/ml compared to input.

#The detection limit of this bioassay was 0.6 log TCI units/ml or 4 TCI units/ml (1.5 log ID<sub>50</sub> units/ml calculated by the method of Reed and Muench [21]).

¹In parentheses prion infectivity titers are given in log ID<sub>50</sub> units/ml as calculated by the method of Reed and Muench [21]

Experiments were performed with mouse scrapie brain homogenate RML5 that had been previously titered in a mouse bioassay. Sensitivity of SCEPA was approximately 0.5 logs less sensitive than the typical mouse bioassay.
Table 2: Prion infectivity in the different fractions after step gradient ultracentrifugation measured by mouse bioassay.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Experiment 1</th>
<th>log Reduction†</th>
<th>Experiment 2</th>
<th>log Reduction†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpi +/- Std</td>
<td>log LD&lt;sub&gt;50&lt;/sub&gt;/ml</td>
<td>dpi +/- Std</td>
<td>log LD&lt;sub&gt;50&lt;/sub&gt;/ml</td>
</tr>
<tr>
<td>Input‡</td>
<td>55 +/- 1</td>
<td>6.6</td>
<td>55 +/- 1</td>
<td>6.6</td>
</tr>
<tr>
<td>T1</td>
<td>104 +/- 8</td>
<td>2.3</td>
<td>96 +/- 10</td>
<td>3.0</td>
</tr>
<tr>
<td>T2</td>
<td>104 +/- 6</td>
<td>2.3</td>
<td>92 +/- 3</td>
<td>3.4</td>
</tr>
<tr>
<td>I</td>
<td>88 +/- 2</td>
<td>3.7</td>
<td>84 +/- 4</td>
<td>4.1</td>
</tr>
<tr>
<td>B</td>
<td>97 +/- 4</td>
<td>3.0</td>
<td>89 +/- 3</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Prion infectivity titer was determined by i.c. inoculation of samples in tga20 indicator mice and subsequent monitoring for development of prion disease. Four mice per group were utilized.

‡Top fraction before ultracentrifugation
† Reduction of prion infectivity titer in log LD<sub>50</sub>/ml compared to input.

Experiments were performed with mouse scrapie brain homogenate RML5 that had been previously titrated in a mouse bioassay.
* infectious prion (native)
~ inactivated prion (denatured)

top fraction (T) containing prions
intermediate fraction (I)
bottom fraction (B)

before ultracentrifugation
during ultracentrifugation
after ultracentrifugation

T1
T2

Figure 1
### Figure 3

#### A

<table>
<thead>
<tr>
<th></th>
<th>short exposure</th>
<th>long exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK</td>
<td>+ + + +</td>
<td>- + -</td>
</tr>
<tr>
<td></td>
<td>T1 T2 I B</td>
<td>B lpt lpt</td>
</tr>
<tr>
<td>MW</td>
<td>75 50 37</td>
<td>75 50 37</td>
</tr>
</tbody>
</table>

#### B

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTA</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>PK</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td></td>
<td>T1 T2 I B</td>
<td>lpt lpt</td>
</tr>
<tr>
<td>MW</td>
<td>75 50 37</td>
<td>75 50 37</td>
</tr>
</tbody>
</table>