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Acetone Precipitation of the Scrapie Agent Results in Successful Recovery of PrP^{Sc} but Decreased Infectivity

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ABSTRACT: Bioassay is considered the most sensitive method for evaluating prion inactivation procedures. Because prions are resistant to methods effective at inactivating conventional microorganisms, prion inactivation research has focused on relatively harsh alternatives, such as concentrated sodium hypochlorite or sodium hydroxide. Often, bioassay for residual infectivity in these studies requires dilution or biochemical alteration of the treated sample in order to maintain subject health and survival. Ideally, prions from treated samples could be sufficiently separated from the inactivating agent without alteration of the sample and with negligible loss of infectivity prior to inoculation into the bioassay host. The current study was designed to evaluate acetone precipitation of the disease-associated form of the prion protein (PrP^{Sc}) from brain homogenate derived from mice with the RML (Rocky Mountain Laboratory) strain of scrapie. The ability to recover PrP^{Sc} was evaluated by Western blot. Dilutions of acetone-precipitated RML-positive brain homogenate were compared to nonprecipitated RML homogenate, resulting in similar PrP^{Sc} detection levels down to 0.025 mg equivalents of brain tissue. The impact of the method on infectivity was investigated by bioassay in intracranially inoculated *tga20* mice. Additionally, contributions to infectivity from the pellet and supernatant fractions were investigated. Acetone precipitation resulted in a 1-$\log_{10}$ reduction in infectivity. Infectivity could not be reconstituted by the acetone soluble fraction of the infectious sample or uninfected brain. This study demonstrates that PrP^{Sc} can successfully be precipitated out of infected brain homogenate using acetone but that there is a reduction in infectivity attributable to the procedure that would need to be considered when evaluating bioassay results.

KEYWORDS: *acetone, bioassay, precipitation, prion, protein, scrapie*

INTRODUCTION

Prions, the causative agents of the transmissible spongiform encephalopathies (TSE), are infectious proteins consisting of an abnormally folded, partially protease resistant isoform of the prion protein (termed PrP^{Sc}).[1] Prions have proven notoriously difficult to inactivate with methods effective against conventional microorganisms, such as ultraviolet irradiation, formalin exposure, and moderate heating.[2] Effective prion decontamination procedures are important both to obviate the risk of human-to-human or zoonotic transmission of TSEs and to aid in controlling the spread of animal TSEs transmitted horizontally, such as scrapie and chronic wasting disease. Additionally, methods of prion inactivation are valuable to the meat processing industry for safe handling and disposal of tissues with the potential to harbor prions, and for the safe production of rendered material used for animal feedstuffs. Decontamination recommendations by the World Health Organization include autoclaving at 134 °C for at least 18 min or up to 1 h, or prolonged (at least1 h) exposure to 1 N sodium hydroxide or ≥20,000 ppm sodium hypochlorite.[3] In prion inactivation studies, procedures necessary for meaningful inactivation may require washing, dilution, or biochemical adjustment of the sample prior to inoculation to avoid adverse effects on bioassay hosts.[4−7] This could potentially give rise to misleading results since the metric for reductions in infectivity are based on increases in incubation time and survival, both of which are affected by the titer of the inoculum. Therefore, it is desirable to have a procedure capable of separating the infectious protein fraction from the potentially detrimental inactivator in a treated sample with minimal or no loss of infectivity.

Standard methods of detecting residual PrP^{Sc} after exposure to candidate inactivating agents include evaluation of treated material by *in vitro* biochemical or immunological assays, including Western blot (WB) and enzyme-linked immunosorbent assay and/or by inoculation of the material into an animal host for *in vivo* bioassay. Molecular techniques such as WB are helpful but alone are insufficient to assess residual infectivity in inactivation studies.[8] Bioassay for residual infectivity in an animal model is the gold standard. Various methods have been used to enrich PrP^{Sc} from tissue samples, mostly to improve WB sensitivity, including precipitation by sodium phosphotungstic acid,[9−11] methanol, ethanol, or trichloroacetic acid[11] as well as concentration by ultracentrifugation. Use of resultant samples from these techniques in bioassays as a means of investigating their value in precipitating PrP^{Sc} from samples containing biologically incompatible substances used in prion inactivation studies has not been reported. Acetone precipitation is a relatively common protein enrichment procedure, but its utility as a method of precipitating PrP^{Sc} from infected tissue samples has not been investigated. In the current study, we report the application of a protein precipitation protocol using acetone to precipitate PrP^{Sc} from mouse brain homogenate of mice

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infected with the RML (Rocky Mountain Laboratory) strain. Western blot was used to evaluate PrP^{Sc} recovery efficacy, and mouse bioassay was used to identify potential impacts of the procedure on infectivity.

MATERIALS AND METHODS

Preparation of Tissue Homogenates. Brain homogenates containing 0.1 mg equivalents of brain tissue/μL (mg equiv/μL) were prepared in Dulbecco's phosphate buffered saline (PBS) from a pool of clinically affected C57BL/6 mice inoculated with the RML strain of scrapie or uninfected C57BL/6 mice. Homogenates were made using a tissue homogenizer (Mini-Beadbeater-8, BioSpec). Briefly, brain tissue was placed inside a sealed polycarbonate tube along with PBS and a small volume of 1.0 mm silica beads. The sample was homogenized for 1 min at 4 °C. This was repeated for a total of 5 homogenizations. Following centrifugation at 4,000*g* for 10 min at 4 °C, the pellet was discarded, and the sample was transferred to a new tube and stored at −80 °C.

Acetone Precipitation of Homogenates. Aliquots of brain homogenate were diluted in PBS to 0.01 mg equiv/μL, mixed with 4 volumes of ice-cold acetone, and incubated overnight at −20 °C. Samples were pelleted (21,000*g* for 10 min at 4 °C), and the supernatant was collected. The pellet was washed in ice-cold acetone for 30 min followed by repeated pelleting. The supernatant was collected, and the pellet was resuspended in PBS to 0.02 mg equiv/μL. Resuspended pellets were stored at −80 °C until analyzed by WB or inoculated into *tga20* mice (see below). For mixed pellet and supernatant experiments, precipitation supernatants were dried down using a vacuum concentrator (Savant SpeedVac, Thermo Scientific). Acetone from the pellet washes was added to desiccated supernatants, and they were dried down again. The resulting residue was resuspended in PBS to a 4× concentration relative to the starting concentration. Pellets and supernatants were reconstituted in the following combinations so the pellet generated a final concentration of 0.01 mg equiv/μL, and the supernatant was brought back to starting concentration (Table 1): (1) acetone-precipitated RML (Ac-RML) pellet + PBS, (2) Ac-RML pellet + Ac-RML supernatant, (3) Ac-RML pellet + supernatant from acetone-precipitated uninfected brain, (4) uninfected brain pellet + Ac-RML supernatant, (5) Ac-RML supernatant in PBS, (6) uninfected brain pellet + uninfected brain supernatant, (7) uninfected brain pellet + PBS, and (8) uninfected brain supernatant in PBS. Samples were stored at −80 °C until analyzed by WB and inoculated into *tga20* mice (see below).

Table 1. Average Survival Times of *tga20* Mice Inoculated Intracranially with Serially Diluted or Acetone-Precipitated RML Scrapie Brain Homogenate

group	experiment	mean survival time in days ± SD[a] (% survival)
	RML Titration Series	
	10^{0}	66 ± 3.4 (0%)
	10^{-1}	74 ± 3.3 (0%)
	10^{-2}	83 ± 6.1 (0%)
	10^{-3}	195 ± 148.5 (22%)
	10^{-4}	394 ± 120.3 (78%)
	10^{-5} through 10^{-12}	455 (100%) − study termination
	Acetone Precipitation	
	Ac-RML[b] 10^{0}	73 ± 6.6 (0%)
	Ac-RML 10^{-1}	94 ± 10.7 (0%)
	Acetone-Precipitated Pellet/Supernatant Combinations	
1	RML/PBS[c]	74 ± 3.5 (0%)
2	RML/RML	79 ± 4.3 (0%)
3	RML/uninfected	77 ± 3.6 (0%)
4	uninfected/RML	483 ± 172.7 (86%)
5	PBS/RML	531 ± 141.3 (90%)
6	uninfected/uninfected	585 (100%) − study termination
7	uninfected/PBS	585 (100%) − study termination
8	PBS/uninfected	585 (100%) − study termination

[a]SD, standard deviation. [b]Ac-RML, acetone-precipitated RML. [c]PBS, phosphate-buffered saline.

Western Blotting. Samples were examined for PrP^{Sc} by WB. Briefly, samples were digested with proteinase K (PK) (USB, Cleveland, OH) at a final concentration of 0.08 mg/mL for 40 min at 48 °C. A protease inhibitor (Pefabloc, Roche, Indianapolis, IN) was added to a final concentration of 0.1 mg/mL to inhibit PK activity. Samples were dissolved in SDS−PAGE sample buffer and analyzed by standard WB procedures. Unless noted, gels were loaded such that 1.0 mg equivalent of brain was loaded for each sample. PrP^{Sc} was detected using monoclonal antibody 6H4 (Prionics, La Vista, NE) at a 1:10,000 (0.1 μg/mL) dilution applied for 1 h at room temperature or 4 °C overnight. A biotinylated sheep antimouse secondary antibody and a streptavidin−horseradish peroxidase (HRP) conjugate (GE Healthcare, Pittsburgh, PA) were used in conjunction with a detection kit (ECL Plus, GE Healthcare) to detect immunolabeling. Secondary antibody and streptavidin−HRP conjugate incubations were conducted at room temperature for 1 h. Blots were developed using a Typhoon 9410 Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA).

Mouse Bioassay. To establish comparative survival curves, a 0.01 mg equiv/μL RML brain homogenate was serially diluted (10-fold dilutions; undiluted or 10^{0} through 10^{-12}), and each dilution was intracranially inoculated into mice of the B6;129S7-$Prnp^{tm1Cwe}$Tg-(Prnp)a20Cwe/CweCnrm (*tga20*) mouse line.[12] For the acetone precipitation experiment, the pellet was resuspended in PBS to 0.01 or 0.001 mg equiv/μL for inoculation. For the mixed pellet and supernatant experiments, the resuspended pellet was combined with an equivalent volume of PBS, or supernatant + PBS, to yield a final concentration of 0.01 mg equiv/μL brain tissue. Supernatant was added as 25% of the total volume to reconstitute the original supernatant concentration. For all inoculation groups, 10 *tga20* mice were anesthetized with isoflurane, and a 30-gauge tuberculin syringe was used to inject 20 μL of brain homogenate into the right cerebral hemisphere at a depth of 3−5 mm. Mice were monitored for 48 h postinoculation for procedure-related adverse events. Mice were then monitored daily and euthanized when they displayed unequivocal neurological signs or at the time of study termination (15 months postinoculation (PI) for the standard survival curve, 19 months PI for the mixed pellet and supernatant experiments). All animal procedures had the approval of the National Animal Disease Center's Animal Care and Use Committee.

Statistics. Kaplan−Meier survival curves were generated using GraphPad Prism, version 4.0 (La Jolla, CA). Survival curves were compared using the logrank test with a level of statistical significance of 0.05. Mice that died within the first 3 weeks of inoculation, presumably due to complications related to intracranial inoculation, or that were removed from the study due to intercurrent disease were censored and not included in survival analyses.

RESULTS

Using a standard protein precipitation technique, we investigated the ability to precipitate PrP^{Sc} from crude brain homogenate using acetone and assayed PrP^{Sc} recovery by WB. Compared to nonprecipitated samples of RML brain homogenate containing equivalent amounts of brain tissue, PrP^{Sc}-immunoreactivity was similarly detectable in precipitated samples containing down to 0.025 mg equivalents of brain tissue (Figure 1). When 0.1 mg equivalents or less were loaded, there was less intense immunoreactivity observed in precipitated versus nonprecipitated samples. Next, we examined the pellet and supernatant fractions of precipitated samples for the

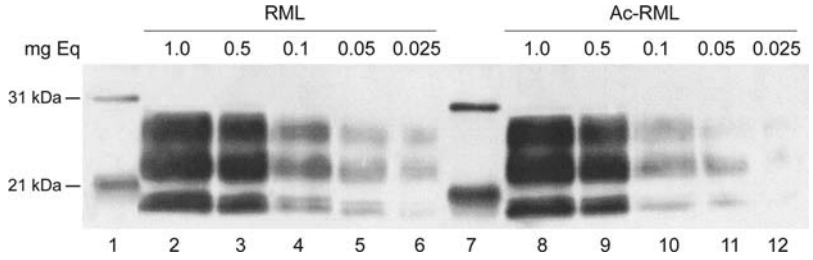

Figure 1. Recovery of protease-resistant PrP^{Sc} by acetone precipitation. Western blot was used to detect PrP^{Sc} in dilutions of nonacetone precipitated (RML) and acetone-precipitated (Ac-RML) brain homogenate from RML infected mice. PrP^{Sc} was detectable in nonprecipitated samples containing down to 0.025 mg equivalents (mg Eq) of brain tissue per lane (lanes 2–6). Similarly, PrP^{Sc} was still detectable in acetone-precipitated samples containing down to 0.025 mg equivalents of brain tissue (lanes 8–12). Lanes 1 and 7, molecular weight marker.

presence of detectable PrP^{Sc}. PrP^{Sc} was detected only in the pellet and not the supernatant or acetone soluble fraction by WB (Figure 2). When bioassayed in *tga20* mice, an approximate

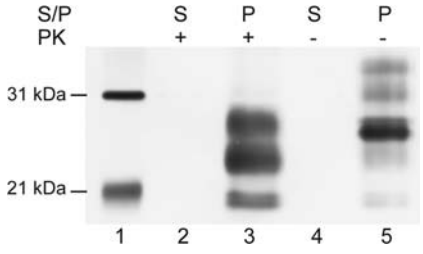

Figure 2. Detection of PrP^{Sc} in the pellet fraction of acetone-precipitated RML brain homogenate. Pellet (P) and supernatant (S) fractions of acetone-precipitated samples were blotted for PrP prior to and after proteinase K (PK) digestion. Protease-resistant PrP^{Sc} was detected in the pellet fraction (lane 3) but not the supernatant (lane 2). PrP was detected in the pellet but not the supernatant prior to PK digestion (lanes 4 and 5). Lane 1, molecular weight marker.

1-$\log_{10}$ reduction in infectivity was observed in acetone-precipitated RML brain homogenate (Ac-RML) reconstituted to contain 0.01 mg equiv/μL of brain tissue (equivalent to the starting dose of RML used to generate the comparative survival curve) (Figure 3). The average survival time for mice inoculated with Ac-RML at 0.01 mg equiv/μL was 73 ± 6.6 days, compared to 66 ± 3.4 days for nonprecipitated samples at 0.01 mg equiv/μL (Table 1). One 10-fold dilution of Ac-RML

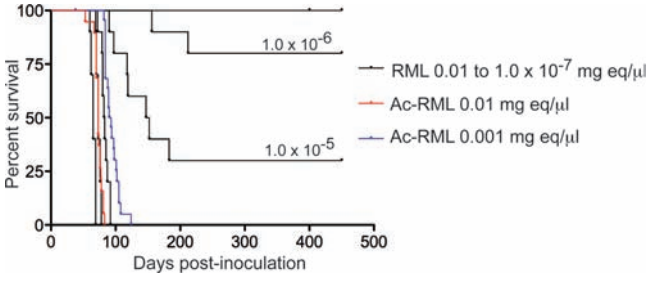

Figure 3. Effect of acetone precipitation on RML infectivity. Kaplan–Meier survival curves were generated to compare acetone-precipitated samples from RML brain homogenate (Ac-RML) containing 0.01 or 0.001 mg equiv/μL of brain tissue with 10-fold serial dilutions of RML scrapie (0.01 to 1.0 × 10^{-7} mg equiv/μL) in *tga20* mice. There was an approximate 1-$\log_{10}$ reduction in infectivity in precipitated samples containing 0.01 mg equiv/μL (red line) and an approximate 2-$\log_{10}$ reduction in precipitated samples containing 0.001 mg equiv/μL (blue line).

to 0.001 mg equiv/μL resulted in an average survival time of 94 ± 10.7 days. As expected, this corresponded to an additional 1-$\log_{10}$ reduction in infectivity (Figure 3).

Next, we assessed reconstitution of Ac-RML pellet and supernatant fractions. Ac-RML pellet and supernatant fractions were reconstituted in various combinations (Table 1). Resuspension of the Ac-RML pellet with Ac-RML supernatant or supernatant from acetone-precipitated uninfected brain resulted in an average survival time of 79 ± 4.3 days and 77 ± 3.6 days, respectively (Table 1). Both corresponded to an approximate 1-$\log_{10}$ reduction in infectivity, similar to the original Ac-RML pellet suspended in PBS (Figures 3 and 4).

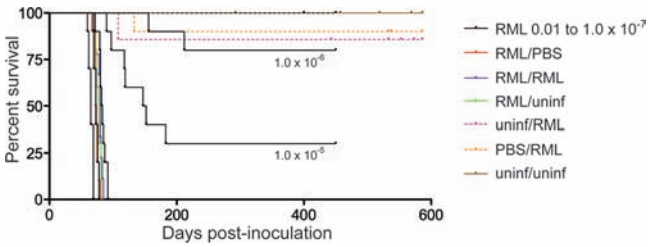

Figure 4. Effects of pellet and supernatant combinations (pellet/supernatant) from acetone-precipitated samples on infectivity. Kaplan–Meier survival curves were generated to compare various acetone-precipitated pellet and supernatant combinations from samples containing 0.01 mg equiv/μL of RML or uninfected brain tissue (uninf) with 10-fold serial dilutions of RML scrapie (0.01 to 1.0 × 10^{-7} mg equiv/μL) in *tga20* mice. Similar to Figure 3, samples containing the precipitated pellet from RML-positive brain reconstituted with either PBS, RML supernatant, or uninfected brain supernatant showed an approximate 1-$\log_{10}$ reduction in infectivity. A single mouse from each group containing the RML supernatant combined with either a pellet from uninfected brain or PBS was euthanized due to clinical disease 100–140 days postinoculation.

One of seven mice in the group inoculated with the combination of acetone-precipitated protein from uninfected mouse brain and Ac-RML supernatant was euthanized at 108 days postinoculation (PI) and confirmed scrapie positive on WB (Figure 4). Similarly, one of ten mice in the group inoculated with PBS combined with Ac-RML supernatant was euthanized at 133 days PI and confirmed scrapie positive. All control mice inoculated with the following combinations survived until study termination at 585 days PI: uninfected brain pellet in PBS, uninfected brain pellet in uninfected brain supernatant, and uninfected brain supernatant in PBS.

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DISCUSSION

An efficacious method of concentrating PrP^{Sc} and separating it from harsh or toxic chemicals used in prion inactivation studies while retaining most if not all infectivity is desirable to allow for adequate bioassay of treatment effectiveness. This study evaluated the effectiveness of acetone precipitation to recover PrP^{Sc} from RML-positive brain homogenate and investigated its impact on infectivity. The presence or absence of PrP^{Sc} in pellet and supernatant fractions was investigated, and recovery was evaluated by comparing serial dilutions of precipitated samples to nonprecipitated samples by WB. Overall, comparable levels of PrP^{Sc} were detected in acetone-precipitated versus non-precipitated samples. PrP^{Sc} was detected in the pellet fraction only and was similarly detectable to nonprecipitated samples down to 0.5 mg equivalents of brain tissue. PrP^{Sc} was still detectable in Ac-RML samples containing 0.1, 0.05, and 0.025 mg equivalents of brain tissue, but immunoreactivity for all bands was less intense versus nonprecipitated samples. This may indicate a partial loss of detectable PrP^{Sc} at these concentrations. Possible mechanisms for this include incomplete pelleting of the sample, reduced solubility following precipitation, or increased proteinase K sensitivity of PrP^{Sc} after exposure to acetone. Evidence for incomplete pelleting was present in pellet/supernatant combination groups that combined the protein pellet from uninfected mouse brain or PBS with Ac-RML supernatant. One animal in each of these groups was euthanized after displaying unequivocal neurologic signs and was subsequently confirmed positive for scrapie.

Because of the procedure's potential value in precipitating PrP^{Sc} from samples containing substances that may be deleterious to the CNS in prion inactivation studies, we also characterized its effect on infectivity by bioassay of Ac-RML samples in *tga20* mice. Acetone precipitation of RML-positive brain homogenate resulted in a 1-$\log_{10}$ reduction in infectivity that could not be reconstituted when the pellet was recombined with its original supernatant or supernatant from pelleting of uninfected mouse brain. This suggests a true partial inactivation of PrP^{Sc} by the procedure versus loss of infectious material. Loss of an essential cofactor in the acetone soluble fraction cannot be excluded, but if so, the loss is irreversible based upon the results from reconstitution experiments. Treatments that disrupt lipid membranes, such as organic solvents or high concentrations of detergents, can reduce scrapie infectivity.[2,6,13,14] Published reports on the effect of acetone extraction on scrapie infectivity are few and predate the understanding of the prion agent as an infectious agent and WB detection of PrP^{Sc}. Consequently, the experimental design of these studies focuses on the acetone soluble extract of brain homogenate rather than the acetone precipitated fraction studied here. In the first study, two goats that were inoculated intracranially with acetone-extracted brain positive for the scrapie agent were free from disease after two years of observation.[15] An indication of residual infectivity in the acetone insoluble fraction is noted in this study based on the observation that an ether extraction of the acetone insoluble fraction produced disease after a prolonged incubation. A reduction in infectivity of 98−99% was demonstrated in mice after inoculation with an acetone and ether extraction of a mouse-passaged strain of scrapie.[16] The acetone only extract was not investigated by bioassay in this study, but the observed decrease in infectivity for combined acetone and ether extraction was similar to the 1-$\log_{10}$ or 99% reduction in infectivity that we observed for acetone alone. In a bioassay application, acetone precipitation would need to be validated using the prion strain of interest as prion strains may differ in their sensitivity to conditions such as pH and temperature variation and exposure to SDS.[17]

The results of this study demonstrate acetone precipitation is an effective method for recovering PrP^{Sc} from TSE brain homogenate, allowing for isolation of residual infectious material without extraneous tissue or chemical contaminants. However, this method did result in a reduction in infectivity that must be considered with regard to its application in bioassay assessment of prion inactivation.

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Notes

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