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ABSTRACT

Studies examining the persistence of prions (the etiological agent of transmissible spongiform encephalopathies) in soil require accurate quantification of pathogenic prion protein (PrP^{TSE}) extracted from or in the presence of soil particles. Here, we demonstrate that natural organic matter (NOM) in soil impacts PrP^{TSE} detection by immunoblotting. Methods commonly used to extract PrP^{TSE} from soils release substantial amounts of NOM, and NOM inhibited PrP^{TSE} immunoblot signal. The degree of immunoblot interference increased with increasing NOM concentration and decreasing NOM polarity. Humic substances affected immunoblot detection of prion protein from both deer and hamsters. We also establish that after interaction with humic acid, PrP^{TSE} remains infectious to hamsters inoculated intracerebrally, and humic acid appeared to slow disease progression. These results provide evidence for interactions between PrP^{TSE} and humic substances that influence both accurate measurement of PrP^{TSE} in soil and disease transmission.

1. Introduction

Prions are the infectious agents in the class of fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs), or prion diseases. TSEs affect a variety of mammals and include bovine spongiform encephalopathy, sheep scrapie, chronic wasting disease (CWD) of deer, elk and moose, transmissible mink encephalopathy of farmed mink, and Creutzfeldt-Jakob disease in humans. While the infectious agent has not been fully characterized, available evidence points to an abnormally folded form of the prion protein (PrP), designated PrP^{TSE}, as the main, if not sole, component of the prion. PrP^{TSE} is formed by the misfolding of normal cellular prion protein, PrP^{C}. The disease-associated form exhibits biophysical properties not shared by PrP^{C} including resistance to proteolysis and inactivation by chemical and thermal treatments, detergent insolubility, and a propensity to form structured aggregates (Colby and Prusiner, 2011). Few microorganisms appear capable of degrading PrP^{TSE} (Booth et al., 2013).

Environmental routes of transmission appear to contribute to scrapie and CWD epizootics, and a growing body of evidence suggests soil may serve as a reservoir of prions in the environment (Pedersen and Somerville, 2012; Schramm et al., 2006). While TSE infectivity is known to persist in soil for at least several years (Brown and Gajdusek, 1991; Seidel et al., 2007), prion concentrations in TSE-endemic areas remains largely unknown. Adequate risk assessments of contaminated environments are currently lacking and require quantitative methods to detect prions in or extracted from natural soils.

Laboratory studies designed to examine prion adsorption to and persistence in soils typically rely on extraction of PrP^{TSE} from soil particles followed by immunodetection (e.g., immunoblotting, enzyme-linked immunosorbent assay) as the primary means of measurement (Cooke et al., 2007; Huang et al., 2007; Jacobson et al., 2009, 2010; Johnson et al., 2006; Leita et al., 2006; Ma et al., 2007; Maddison et al., 2010; Russo et al., 2009; Seidel et al., 2007). To date, effective elution of PrP^{TSE} from soil particles has been accomplished only with anionic detergents such as sodium dodecyl sulfate (SDS) or sodium N-lauroylsarconsinate (sarkosyl)

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(Cooke et al., 2007; Johnson et al., 2006; Seidel et al., 2007). Direct detection of soil-bound prions using antibody-based techniques has also been reported (Genovesi et al., 2007; Saunders et al., 2009). Relatively few studies have employed protein misfolding cyclic amplification (PMCA) (Russo et al., 2009; Saunders et al., 2011a,b,c; Seidel et al., 2007) or animal infectivity assay (Brown and Gajdusek, 1991; Johnson et al., 2006, 2007, 2011a; Saunders et al., 2011a; Seidel et al., 2007). The relative merits of these detection methods in environmental studies have been discussed elsewhere (Smith et al., 2011). In this contribution, we focus on immunoblotting and animal bioassay. The former ranks among the most widely used detection methods in experimental studies; the latter is typically considered the "gold standard" for prion detection.

While recent studies have examined PrP^{TSE} attachment to soils varying in organic carbon content (Cooke et al., 2007; Johnson et al., 2006; Maddison et al., 2010), the influence of natural organic matter (NOM) on PrP^{TSE} measurement in soil or other environmental matrices has not been specifically investigated. Incomplete recovery and difficulty in separating proteins from co-extracted constituents of the soil matrix can complicate accurate quantification of proteins in soils. Soil enzymes (e.g., urease, phenol oxidases, proteases, hydrolases) and the glycoprotein glomalin produced by arbuscular mycorrhizal fungi are extracted from soil simultaneously with NOM (Boyd and Mortland, 1990; Schindler et al., 2007). The presence of NOM in soil extracts can interfere with accurate protein detection by SDS polyacrylamide gel electrophoresis (PAGE) and common total protein assays (viz. Bradford, Lowry, and bicinchoninic acid assays) (Murase et al., 2003; Roberts and Jones, 2008; Rosier et al., 2006).

The objective of this study was to determine the extent to which NOM influences PrP^{TSE} measurement by immunoblotting and animal bioassay. We determined the amount of NOM co-extracted with PrP^{TSE} from soil and compost samples by common PrP^{TSE} extraction methods. The impact of NOM on immunoblot detection of PrP^{TSE} was determined by spiking the protein into soil extracts or solutions of humic substances. Several methods were evaluated for their potential to remove NOM from PrP^{TSE} samples. The influence of NOM on prion detection by animal bioassay was assessed by intracerebrally inoculating Syrian hamsters with PrP^{TSE} that had been allowed to interact with humic acid.

2. Materials and methods

2.1. Prion protein sources

The HY strain of hamster-adapted transmissible mink encephalopathy and the CWD agent were obtained from brain tissue of experimentally inoculated Syrian hamsters and white-tailed deer (Johnson et al., 2011b). Infected hamster and deer brain tissues were homogenized (10% w/v) in PBS and stored at −80 °C until use. Most experiments employed PrP^{TSE} purified to a P4 pellet by the procedure of Bolton et al. (1987) modified by excluding proteinase K digestion (McKenzie et al., 1998). The P4 pellet isolated from four hamster brains was resuspended in 1 mL of 10 mM tris(hydroxymethyl)aminomethane (Tris; pH 7.4) with 130 mM NaCl. The resulting protein concentration was determined using the Pierce BCA protein assay as directed by the manufacturer's instructions. PrP^{TSE} concentration was taken as 87% of the total protein (Silveira et al., 2005). A subset of experiments employed brain homogenates (BHs) treated with proteinase K (PK) prepared by incubating homogenized tissue with 50 μg mL^{-1} PK (1 h, 37 °C). PK activity was then inhibited by addition of phenylmethylsulfonyl fluoride to a final concentration of 4 mM. Purified, full-length (23–230) recombinant murine PrP in an α-helix-rich conformation (α-recPrP) similar to that of PrP^{C} was produced as previously described (Colby et al., 2007).

2.2. NOM sources

Humic acids from Elliot soil (ESHA, 1S102H), the Suwannee River (SRHA, 2S101H), Pahokee peat (PPHA, 1S103H), Leonardite (LHA, 1S104H) and fulvic acid from Elliot soil (ESFA, 1S102F) were purchased from the International Humic Substances Society (IHSS; St. Paul, Minnesota, USA) and used without further purification. Selected properties of the humic substances are presented in Supplementary Table 1 (Thorn et al., 1989; Ritchie and Perdue, 2003). Stock solutions were prepared by dissolving humic or fulvic acid in minimal quantities of 0.01 M NaOH and diluting to 2 mg mL^{-1} (final concentration) with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.0) in 10 mM NaCl.

Pahokee peat soil and Elliot silt loam soil were purchased from IHSS, and composted beef cattle manure amended with sawdust was provided by Shannon Bartlet-Hunt (University of Nebraska). Selected physicochemical properties of the soils are presented in Supplementary Table 2.

Soil extracts were prepared using previously reported detergent-based extractants for PrP^{TSE} (Cooke et al., 2007; Johnson et al., 2006; Seidel et al., 2007). Soil and compost samples (25 mg) were extracted with 100 μL distilled deionized water (ddH_2O, 18 MΩ-cm resistivity; 1 h, 22 °C), 1% SDS in ddH_2O (1 h, 22 °C), 1% (w/v) sarkosyl in 100 mM sodium phosphate buffer (pH 7.4; 1 h, 37 °C), or 10× SDS-PAGE sample buffer (100 mM Tris, 7.5 mM EDTA, 100 mM dithiothreitol (DTT), 350 mM SDS, pH 8.0; 10 min, 100 °C). The suspensions were centrifuged (10 min, 1000*g*), and supernatants were saved for experiments with PrP^{TSE}.

2.3. Estimation of NOM concentration in soil and compost extracts

NOM concentrations in soil and compost extracts were estimated by UV–vis absorption because the large concentrations of detergents in extracts made accurate determination of DOC concentrations difficult by high temperature combustion or UV/persulfate oxidation. Absorbance spectra (250–700 nm) were acquired using a UV-3600 Shimadzu spectrophotometer. In preliminary experiments, we compared estimation of NOM concentrations in Elliot soil extracts using absorbance at $\lambda = 254$, 465, and 665 nm and obtained equivalent results. Subsequent analysis employed absorbance at $\lambda = 465$ nm using a Spectra Max Plus microplate reader (Molecular Devices, Inc.). Triplicate samples were quantified against a five-point calibration curve ($R^2 > 0.98$) produced with 0.005–1 g L^{-1} ESHA for Elliot soil and compost extracts, or PPHA for Pahokee peat soil extracts. Humic acid standards were prepared in the same solutions used for soil extraction. Samples with absorbances outside the linear range of the standard curve were diluted and reanalyzed.

2.4. Immunoblot analysis

To determine the effect of soil and composts extracts on PrP^{TSE} detection by immunoblotting, 0.1 μg purified PrP^{TSE} was mixed thoroughly with each soil or compost extract (20 μL), incubated for 1 h, and prepared for analysis by SDS-PAGE with immunoblot detection. The effect of humic substances on immunoblot detection of PrP^{TSE} was investigated by mixing 0.1 μg purified PrP^{TSE} with 20 μL of 0.05–1 mg mL^{-1} humic substance solutions in 10 mM HEPES, 10 mM NaCl, pH 7.0. For experiments examining the influence of polyphenolic compounds on PrP^{TSE} immunoblotting, PrP^{TSE} (0.1 μg) was mixed with 20 μL of 1 mg mL^{-1} tannic acid, epigallocatechin gallate (EGCG), katacine, or rutin and incubated for 1 h prior to

analysis by SDS-PAGE with immunoblot detection. All experiments were replicated multiple times. The immunoblots presented in the figures are representative of triplicate experiments for which relative intensities were determined by densitometry.

Protein precipitation by methanol or sodium phosphotungstic acid (NaPTA) (Sigma) was examined as a potential method to separate PrP^{TSE} from NOM. For methanol precipitation, protein was precipitated using four volumes of cold methanol (4 °C) and sedimented by centrifugation at 24,000*g* for 30 min. The resulting protein pellets were resuspended in 1× NuPAGE lithium dodecyl sulfate (LDS) sample buffer (30 μL) for subsequent analysis. For NaPTA precipitation, PrP^{TSE} samples were mixed with an equal volume of 4% (w/v) sarkosyl in PBS. NaPTA stock solution (4% (w/v) NaPTA, 170 mM $MgCl_2$ in ddH_2O adjusted to pH 7.0 with 1 M NaOH) was added to a final concentration of 0.3% (w/v). The samples were incubated with shaking (1 h, 37 °C) and centrifuged (14,000*g*). The resulting pellets were resuspended in 1× NuPAGE sample buffer (30 μL). All protein samples were prepared for NuPAGE by adding LDS sample buffer (7.5 μL) and NuPAGE sample reducing agent (3 μL) containing 500 mM DTT (Invitrogen), and heating (10 min, 70 °C). Protein samples (32 μL) were fractionated on 12% bis–tris polyacrylamide gels (Invitrogen) and electrotransferred to polyvinyl difluoride (PVDF) membranes. Membranes were blocked in 5% non-fat dry milk in Tris buffered saline containing 0.1% Tween 20 (overnight, 4 °C). Hamster PrP was immunoblotted with monoclonal antibody (mAb) 3F4 (1:40,000 dilution), mAb SAF 83 (1:200 dilution, Cayman Chemical), or mAb 8B4 (1:1000 dilution, Santa Cruz Biotechnology); deer PrP was probed with mAb 6D11 (1:1000 dilution, Covance). We probed α-recPrP with mAb 8G8 (1:1000 dilution, Cayman Chemical). Detection was achieved with HRP-conjugated goat anti-mouse immunoglobulin G (1:10,000; Bio-Rad) and Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). The intensity of immunoreactivity in blots was measured using density histograms of the protein bands and converted to intensity relative to the humic acid-free control (ImageJ). In the figures and associated discussion, we report mean relative intensities of triplicate experiments and the associated error as the standard deviation. To demonstrate the reproducibility of our results, we present a sample of our replicate data Supplementary Fig. 1. Supplementary Fig. 2a graphically shows the reproducibility of the densitometry measurements across a range of concentrations for two humic acids. Supplementary Fig. 2b plots replicate densitometry data for PrP^{TSE} incubated with a single concentration of five humic acids.

2.5. Infectivity assay

Purified PrP^{TSE} (2 or 0.2 μg) was incubated with 0.25 mg mL^{-1} ESHA or SRHA as described above and resuspended in 10% (w/v) BH prepared (20 μL) from uninfected hamster brain tissue in PBS. PrP^{TSE}-HA samples were intracerebrally inoculated into male weanling Syrian hamsters (Harlan, Indianapolis, Indiana, USA). Equivalent amounts of PrP^{TSE}, ESHA, or SRHA were inoculated into control animals. Hamsters were observed twice weekly for the onset of clinical signs then monitored twice daily to assess disease progression (Bessen and Marsh, 1992). All animal work was conducted with approval of National Wildlife Health Center institutional animal care and use committee. Times to onset of clinical signs in infectivity assays were modeled as a function of PrP^{TSE} dose (2 vs. 0.2 μg) and humic acid type (ESHA or SRHA vs. none). The models were log-linear; logarithms were taken of all quantities prior to linear least squares fitting. Coefficient estimates and their 95% confidence intervals ($CI_{95\%}$) were re-exponentiated to produce estimated multipliers on the raw scale.

For hamster scrapie agent, infectious titer strongly correlates with time to onset of clinical signs of disease after intracerebral inoculation (Prusiner et al., 1982). Extension of this relationship to our study relied on two assumptions: (i) HY agent exhibits a relationship between titer and incubation period similar to that of 263 K, and (ii) the log-linear relationship also holds for a purified preparation of PrP^{TSE}. Further discussion of these assumptions is provided in the Supplementary Data.

3. Results

3.1. Estimation of NOM concentration in soil and compost extracts

To determine the extent PrP^{TSE} extraction conditions co-extract NOM, we estimated the amount of chromophoric organic matter released from Elliot soil, Pahokee peat soil, and beef cattle manure compost when extracted with water or anionic detergent solutions previously employed to extract PrP^{TSE} from soil (Table 1). As expected, detergent extracts of the soils and compost contained higher NOM concentrations than did water extracts. SDS sample buffer (10 min, 100 °C) extracts of Pahokee peat soil and compost contained ~8300 and ~1200 μg mL^{-1} NOM, respectively. Extraction with SDS sample buffer released ~500 μg mL^{-1} NOM from Elliot soil. The lower amounts of NOM extracted from Elliot soil than from Pahokee peat soil by all detergent extracts tested is consonant with the organic carbon contents of the soils (45.7% for Pahokee peat soil, 2.9% for Elliot soil; Supplementary Table 2). We note that specific extinction coefficients (ε_i, where *i* is wavelength in nm) vary among NOM samples (e.g., $\varepsilon_{465} = 6.05$ and 3.73 L g^{-1} cm^{-1} for ESHA and PPHA in water, respectively); the reported NOM concentrations must therefore be viewed as approximations.

3.2. NOM interferes with immunoblot detection of PrP^{TSE}

The influence of co-extracted NOM in SDS sample buffer extracts of each soil and compost on PrP^{TSE} immunoblotting is presented in Fig. 1. Immunoblot signals were dramatically reduced for PrP^{TSE} that had been incubated with soil and compost extracts. Immunoblot signals for PrP^{TSE} that had been incubated with soil extracts appeared at the expected position in the gel; no streaking of PrP immunoreactivity was observed. Electrophoresis of samples containing NOM produced visible brown streaks within the polyacrylamide gel, which were mostly retained in the gel after electroblotting. Using mAb 3F4, the PrP^{TSE} signal intensity in Elliot and Pahokee peat soil extracts was reduced to 50% and 8% of that of the protein in SDS sample buffer, respectively. Incubation with compost extract reduced the PrP^{TSE} signal to 9% of that of the protein in SDS sample buffer. These extracts were prepared by heating

Table 1
Estimated concentrations of NOM in soil and compost extracts.

Extractant[b]	NOM concentration (μg mL^{-1})[a]		
	Elliot soil	Pahokee peat soil	Compost
ddH_2O	120 ± 16	130 ± 11	380 ± 16
1% SDS	220 ± 30	840 ± 29	380 ± 54
1% Sarkosyl	151 ± 3	6100 ± 160	530 ± 35
SDS sample buffer	500 ± 22	8300 ± 830	1200 ± 40

[a] The concentration of chromophoric NOM extracted was estimated by UV–vis absorbance at 465 nm using standard curves prepared with known concentrations of ESHA (Elliot soil and compost) or PPHA (Pahokee peat soil). Values are means of three replicate samples, and error represents one standard deviation.

[b] Samples were extracted with ddH_2O or 1% SDS for 1 h at room temperature, 1% sarkosyl in 100 mM sodium phosphate buffer (pH 7.4) for 1 h at 37 °C, or 5 × SDS-PAGE sample buffer (100 mM Tris, 7.5 mM EDTA, 100 mM DTT, 350 mM SDS pH 8.0) at 100 °C for 10 min.

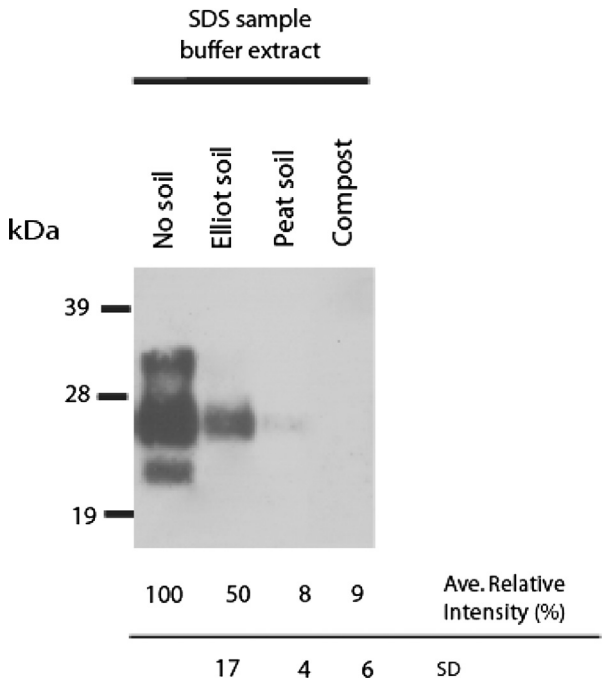

Fig. 1. Natural organic matter interferes with PrP detection by immunoblotting. Natural organic matter present in extracts of Elliot soil, Pahokee peat soil and compost inhibited detection of PrP^{TSE} by immunoblotting. Soil and compost samples were extracted with SDS-PAGE sample buffer for 10 min at 100 °C. Estimated amounts of NOM in the sample extracts are presented in Table 1. An equivalent amount of purified PrP^{TSE} (0.1 μg) was added to the soil and compost extracts as for the lane labeled PrP^{TSE}. The reduction in PrP^{TSE} levels was estimated by densitometry. The mean relative intensity of experimental triplicates with a standard deviation (SD) was determined for each protein signal.

suspensions of the soils in SDS sample buffer to 100 °C for 10 min, a treatment abolishing proteolytic activity. To account for the unlikely possibility that heat-denatured proteases regained activity upon cooling, we added PrP^{TSE} directly to soil extracts at 100 °C and immunoblotted immediately. We observed equivalent reductions in PrP^{TSE} immunoblot signals as above (data not shown) indicating signal loss was not attributable to proteolytic degradation.

To further investigate the nature of immunoblot interference by NOM, we examined the effect of soil humic acid on PrP^{TSE} immunoblot signal. We selected ESHA and SRHA because they differ considerably in polarity, acidic functional group content, and aromaticity (Supplementary Table 1). Purified PrP^{TSE} was incubated with 0.05–1 mg mL^{-1} ESHA or SRHA (Fig. 2). The intensity of the PrP^{TSE} immunosignal decreased with increasing concentrations of either ESHA or SRHA; however, SRHA reduced PrP^{TSE} immunoblot signal to a much smaller extent. The PrP^{TSE} immunoblot signal decreased to 54% and 10% of that of the starting material in the presence of 1 mg mL^{-1} SRHA and ESHA, respectively. Contaminant proteases in the humic solutions did not appear responsible for the loss of PrP^{TSE} immunoblot signal, as treatments to inactivate proteolytic activity did not prevent this effect (Supplementary Fig. 3). The potential effect of NOM polarity on PrP^{TSE} immunoblotting was further examined with three additional humic substances spanning a broader range of polarities: LHA, PPHA, and ESFA (Supplementary Table 1). Immunoreactivity of PrP^{TSE} decreased to 1%, 20% and 91% of the intensity of the starting material in the presence of 1 mg mL^{-1} LHA, PPHA and ESFA, respectively (Fig. 2).

We then tested if humic substances interfered with immunoblot detection by antibodies directed against different segments of the PrP primary sequence. The results described above were obtained using monoclonal antibody (mAb) 3F4, which binds to residues 110–113 (MKHM) in hamster PrP. Immunoblots probed with antibodies directed against different segments of the PrP primary sequence also exhibited signal reduction in the presence of ESHA (Fig. 3). These experiments used mAb SAF 83, which has an epitope within residues 126–164, and mAb 8B4, which recognizes residues 37–39 (RYP) in the N-terminal domain. In immunoblots probed with SAF 83, PrP^{TSE} incubation with 1 mg mL^{-1} ESHA substantially reduced the signal when compared to PrP^{TSE} in the absence of humic acid. The immunoblot signal of PrP^{TSE} probed by mAb 8B4 was completely inhibited by the presence of 0.25–1 mg mL^{-1} ESHA (Fig. 3). The N-terminus of PrP^{TSE} (~70 residues) is susceptible to proteolytic cleavage, which would prevent detection by mAb 8B4. However, treatment of ESHA with protease inhibitors or heating to 100 °C, as described above, did not restore PrP^{TSE} signal with mAb 8B4 (Supplementary Fig. 3).

3.3. *ESHA interferes with immunoblot detection of cervid PrP^{TSE} and α-recPrP*

We then examined whether NOM interferes with immunoblot detection of PrP^{TSE} when present in the complex matrix of brain homogenate and if the interference occurred for PrP^{TSE} from another species. We incubated brain homogenates from hamsters and deer infected with the HY strain and CWD, respectively, with 1 mg·mL^{-1} ESHA and immunoblotted (Fig. 4). Prior to incubation with ESHA, the brain homogenates were treated with proteinase K (PK) to remove any contribution from PrP^{C} to immunoreactivity. Treatment with PK also cleaves the N-terminal ~70 amino acid residues from PrP^{TSE}. Immunoblot signals for PrP^{TSE} in both hamster and deer brain homogenate were inhibited by ESHA despite the large background of proteins and other biomolecules in the brain homogenate (Fig. 4). Densitometric measurements revealed PrP^{TSE} signals were reduced to 33% and 21% for the hamster and deer brain homogenates, respectively.

To test whether the interference of immunoblot detection by NOM was specific to the pathogenic conformation of prion protein, we examined the influence of ESHA on immunoblotting of full-length recombinant murine PrP. The primary sequences of the mature mouse and hamster PrP exhibit 12 site differences (Supplementary Fig. 4). The α-recPrP used in this study differs from PrP^{TSE} in that it lacks N-linked glycans and the glycosylphosphatidylinositol (GPI) anchor, has a predominantly α-helical secondary structure resembling PrP^{C}, and is soluble and monomeric in aqueous solution. Incubation with 1 mg·mL^{-1} ESHA reduced the α-recPrP immunosignal to 14% of that of the starting material (Fig. 4).

3.4. *Polyphenolic compounds influence immunoblot detection of PrP^{TSE}*

To investigate the possible contribution of polyphenol and glycoside moieties to NOM interference of PrP^{TSE} immunoblotting, we tested four compounds containing functionalities similar to those found in humic substances: tannic acid, rutin, katacine, and epigallocatechin gallate (EGCG) (Fig. 5; *cf.* Supplementary Fig. 5 for structures). Interestingly, 1 mg mL^{-1} EGCG substantially reduced PrP^{TSE} immunoblot signal, and the PrP^{TSE} signal was completely inhibited in the presence of 1 mg mL^{-1} tannic acid. Rutin and katacine diminished PrP^{TSE} immunoblot signal intensity to a lesser extent.

3.5. *Evaluation of protein precipitation and purification methods to isolate PrP^{TSE} from ESHA*

We examined two protein precipitation agents for their ability to separate PrP^{TSE} from ESHA: methanol and NaPTA. Mixtures of

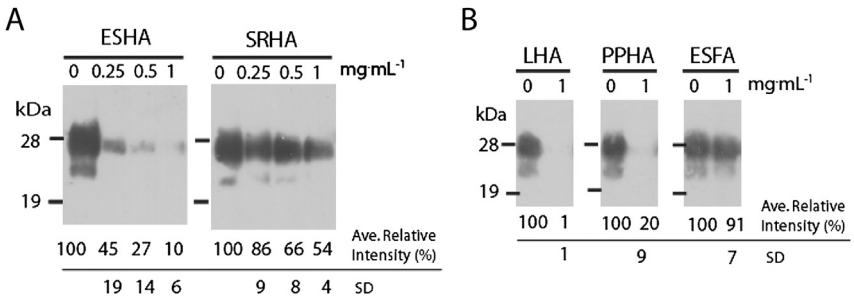

Fig. 2. Humic substances varying in polarity inhibit PrP^{TSE} immunoblots to differing extents. (a) PrP^{TSE} (0.1 μg) was incubated (1 h) with increasing amounts of ESHA and SRHA. (b) LHA, PPHA, and ESFA interfere with PrP^{TSE} immunoblotting. Purified PrP^{TSE} (0.1 μg) was incubated (1 h) with 1 mg mL^{-1} of each humic substance. These experiments were conducted in triplicate. The reduction in PrP^{TSE} levels was estimated as the mean of three replicates with a standard deviation (SD) as indicated. Immunoblots were probed with mAb 3F4. Replicate immunoblots and measurement of the reduction in PrP^{TSE} levels are provided in the Supplementary Figs. 1 and 2.

purified PrP^{TSE} (0.1 μg) and 1 mg mL^{-1} ESHA were treated with cold methanol or NaPTA. In both cases, the resulting protein pellet was colored brown with humic acid. While precipitation partially ameliorated the effect of ESHA, substantial signal inhibition remained. Immunoblotting PrP^{TSE} co-precipitated with ESHA reduced PrP^{TSE} signal by 42% for NaPTA precipitation and 26% for methanol precipitation (Fig. 6). The extent of PrP^{TSE} immunoblot signal suppression by ESHA was less for methanol-precipitated PrP^{TSE} (26%; Fig. 6) than for PrP^{TSE} that was not methanol precipitated (90%; Fig. 2). In the absence of PrP^{TSE}, NaPTA and methanol precipitation conditions did not sediment ESHA (data not shown) suggesting that ESHA co-precipitated with PrP^{TSE} was part of a protein-humic complex. Other methods tested separating PrP^{TSE} from ESHA proved ineffective. These included centrifugal ultrafiltration (Amicon 10 kDa nominal molecular weight cut off, Millipore), selective removal of humic substances by insoluble polyvinylpolypyrrolidone, and selective PrP^{TSE} removal using a ligand-based kit (PAD Beads, CalBioreagents) (data not shown).

3.6. *Effect of humic acid on the effective titer of TSE agent in bioassay*

Animal bioassay is considered the "gold standard" in prion detection. Prion dose is quantified in terms of titer and determined by serial dilution of agent in test animals to determine the median infectious dose (ID_{50}; the dose required to infect half a test population). For hamster scrapie agent, the strong inverse relationship between dose and time to onset of clinical signs of disease after intracerebral inoculation has also been used to estimate titer (Prusiner et al., 1982).

We examined the influence of humic acid on the incubation period of hamsters intracerebrally inoculated with two doses of PrP^{TSE} (Fig. 7; Supplementary Fig. 6). Hamsters inoculated with 2 or 0.2 μg of PrP^{TSE} alone exhibited clinical signs at 78 ± 3 or 91 ± 2 days post inoculation (dpi). Including 0.25 mg mL^{-1} ESHA or SRHA in inocula resulted in incubation periods of 83 ± 5 dpi and 83 ± 3 dpi for ESHA and SRHA with 2 μg PrP^{TSE} and 102 ± 7 dpi and 97 ± 4 dpi for ESHA and SRHA with 0.2 μg PrP^{TSE}. Comparing incubation periods among the 2 μg of PrP^{TSE} treatment groups suggests humic acid caused a very small, but statistically significant ($p = 0.003$ and 0.0001 for ESHA and SRHA) lengthening of the time to onset of clinical signs. In the 0.2 μg PrP^{TSE} treatment group, ESHA and SRHA extended the mean incubation period by at least 11 and 6 days ($p = 0.002$ and 0.004), respectively, indicating a slight reduction in effective titer.

Small changes in titer resulting in incubation periods lengthened by <7 days are not easily interpreted using the time interval assay (Prusiner et al., 1982). We therefore assessed the statistical significance of the potential extension in the incubation period across doses and treatment groups using a log-linear model (Supplementary Table 3). We found ESHA and SRHA caused estimated 9.3% ($CI_{95\%}$: 6.3%, 12.4%) and 6.6% ($CI_{95\%}$: 3.7%, 9.7%) increases in mean incubation periods compared to control, respectively. Our model produced limited evidence for a difference between the effects of ESHA and SRHA on mean incubation periods ($p = 0.09$).

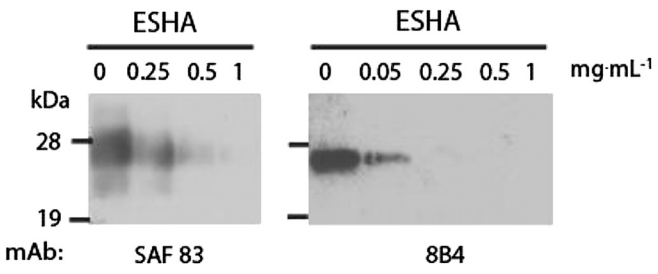

Fig. 3. Prion protein signal was reduced or eliminated by ESHA in immunoblots probed with antibodies directed against different segments of PrP^{TSE}. Purified PrP^{TSE} (0.1 μg) was incubated (1 h) with the indicated concentration of EHSA. Immunoblots were probed with mAb SAF 83 or mAb 8B4 as indicated.

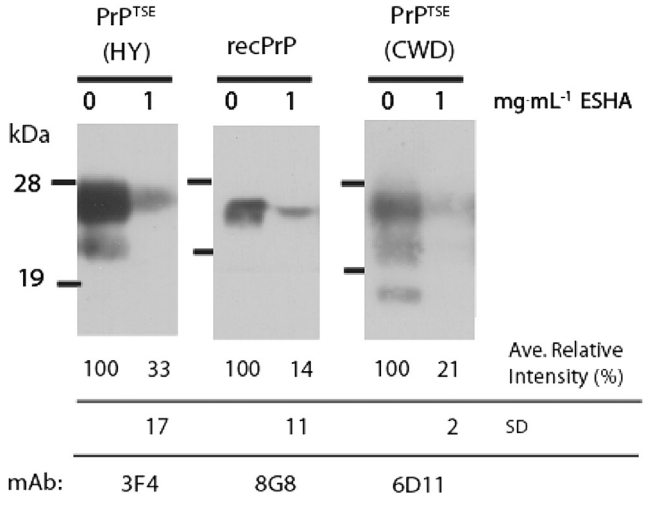

Fig. 4. Elliot soil humic acid inhibited immunoblot detection of PrP^{TSE} in both hamster and deer brain homogenate and recombinant prion protein. Proteinase K (PK)-treated HY-infected hamster BH (10% (w/v); PrP^{TSE}(HY); 4 μL) and CWD-infected deer BH (10% (w/v); PrP^{TSE}(CWD); 4 μL), and recombinant murine prion protein (α-recPrP; 0.5 μg) were incubated (1 h) with ESHA. The reduction in PrP^{TSE} levels was estimated by densitometry as the mean percent relative intensity in experimental triplicates with standard deviation (SD). Immunoblots were probed with mAbs 3F4, 8G8 or 6D11 as indicated.

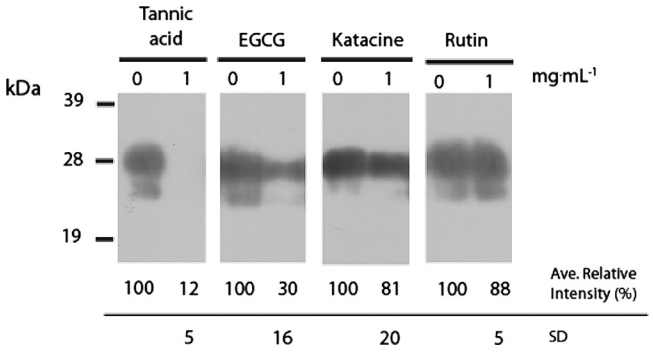

Fig. 5. Polyphenolic compounds inhibit PrP^{TSE} immunoblotting. Purified PrP^{TSE} (0.1 μg) was mixed with 20 μL of 1 mg mL^{-1} tannic acid, EGCG, katacine, or rutin and incubated for 1 h at room temperature. The reduction in PrP^{TSE} levels was estimated by densitometry as the mean percent relative intensity in triplicate experiments with standard deviation (SD). Immunoblots were probed with mAbs 3F4.

4. Discussion

Our results demonstrate that methods effective for extracting PrP^{TSE} from soil also remove NOM, which can interfere with PrP^{TSE} detection by immunoblotting. The extent of interference depended on both the concentration and specific physicochemical properties of NOM. Analysis of five humic substances varying in polarity (based on a polarity index defined as $((O + N + S)/C)$ revealed that interference with PrP^{TSE} immunoblotting decreases as humic substance polarity increases. The extent of interference with PrP^{TSE} immunoblotting was also correlated with humic substance aromaticity; PrP^{TSE} immunoblotting interference increases as the humic substance aromaticity increases (Supplementary Table 1). These results may reflect the contribution of the hydrophobic effect to interaction between PrP^{TSE} and humic substances. Hydrophobic interactions have been suggested to contribute to lysozyme complexation with humic acid (Tan et al., 2009), and interaction with hydrophobic domains in humic substances has been advanced as an explanation for protein preservation in sediment environments (Zang et al., 2000). Polyphenolic compounds (viz. tannic acid and EGCG) were found to reduce PrP^{TSE} immunoblot signal, consistent with the idea that polyphenolic structures within NOM

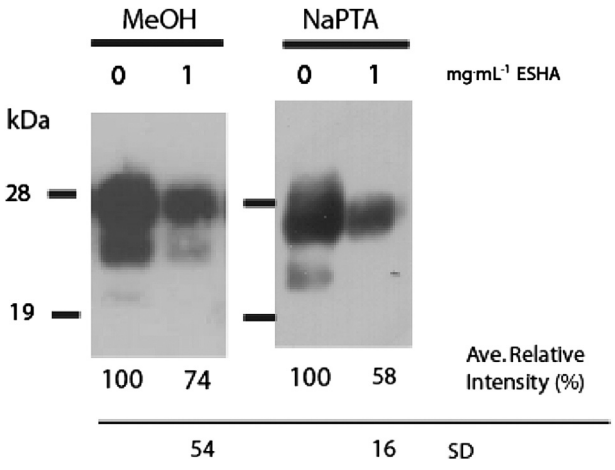

Fig. 6. Elliot soil humic acid co-precipitates with PrP^{TSE}. Mixtures of purified PrP^{TSE} (0.1 μg) and 1 mg mL^{-1} ESHA were precipitated with four volumes of cold methanol, or 0.3% (w/v) sodium phosphotungstic acid (NaPTA), 170 mM $MgCl_2$ in water, pH 7.0. In each immunoblot, the reduction in PrP^{TSE} levels was estimated by densitometry and the percent relative intensity represents the mean of three experiments with indicated standard deviation (SD). Immunoblots were probed with mAbs 3F4.

may contribute to interactions with PrP^{TSE} and interference with PrP^{TSE} immunoblotting. However, this result does not exclude the contribution of other types of NOM molecules to the mechanism of PrP^{TSE} immunoblot interference. Prions have been previously hypothesized to interact directly with naturally occurring polyphenolic compounds including tannic acid and katacine (Kocisko et al., 2003). These compounds inhibit formation of protease-resistant PrP in scrapie-infected neuroblastoma cells at concentrations >1 μM (Kocisko et al., 2003).

We also demonstrated that ESHA interferes with immunoblotting of cervid PrP^{TSE}, rodent PrP^{TSE}, and α-recPrP. These results suggest that NOM interferes with immunoblot detection of prion protein from multiple species and when PrP^{TSE} is present in the complex matrix of brain homogenate. Thus, we expect that immunoblot detection of CWD prions may be influenced by NOM in environmental samples. Since PrP^{TSE} (HY) and PrP^{TSE} (CWD) were treated with PK prior to incubation with ESHA, the N-terminal region of PrP is not necessary for NOM interference. Interference of α-recPrP signal by ESHA suggests the glycosylation, glypiation, and β-sheet structure of PrP^{TSE} are not required for immunoblot interference.

Possible explanations for NOM interference with PrP^{TSE} immunoblotting include (1) occlusion of antibody epitopes; (2) interference with electrotransfer to the PVDF membrane; (3) altered migration in the gel; and (4) limited entry of PrP^{TSE} into the polyacrylamide gel. NOM interference of immunoblotting was found for antibodies directed at different segments of the PrP molecule

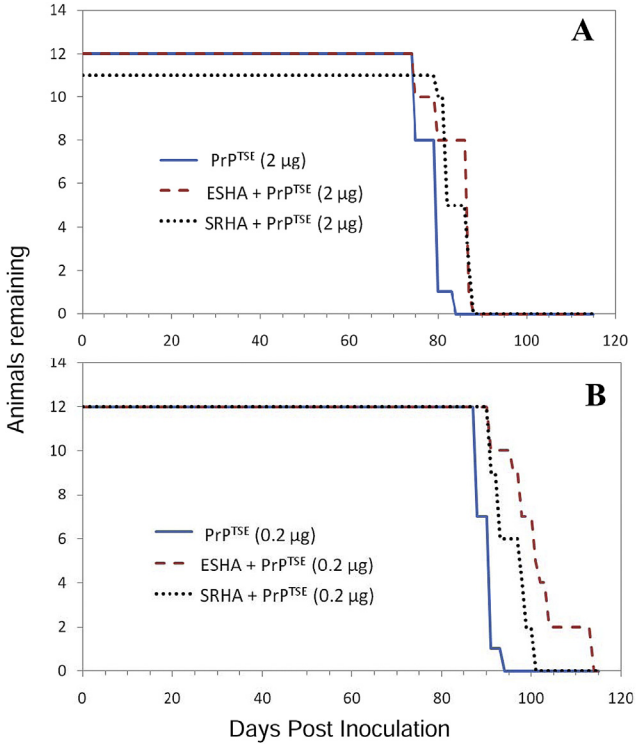

Fig. 7. Humic acids decrease effective prion titer in intracerebral animal bioassays. Prions associated with SRHA and ESHA remain infectious. Two doses of purified PrP^{TSE} (2 μg and 0.2 μg) were incubated with 0.25 mg mL^{-1} ESHA or SRHA for 1 h and intracerebrally inoculated into weanling hamsters. Hamsters inoculated with PrP^{TSE} alone exhibited incubation times of 78 ± 3 and 91 ± 2 dpi for 2 μg and 0.2 μg doses, respectively. PrP^{TSE}-ESHA exhibited incubation times of 83 ± 5 and 102 ± 7 dpi for 2 μg and 0.2 μg doses, respectively. PrP^{TSE}-SRHA exhibited incubation times of 83 ± 3 and 97 ± 4 dpi for 2 μg and 0.2 μg doses, respectively. Hamsters dosed with humic acid alone ($n = 16$; 0.25 mg mL^{-1} ESHA or SRHA) remained healthy throughout the course of the experiment (data not shown).

(Fig. 3) requiring that NOM occlude multiple parts of the PrP molecule for explanation (1) to hold. Electrophoresis of PrP^{TSE} in the presence of NOM produced visible brown streaks within the polyacrylamide gel, which were mostly retained in the gel after electroblotting, suggesting a role for explanation (2). Altered electrophoretic mobility (explanation 3), in the absence of other effects, would result in a smeared signal or a signal appearing at unexpected positions in the gel. This explanation is not consistent with our observation of PrP immunoreactivity appearing only in the expected molecular weight range. However, we cannot rule out diffuse migration of PrP through the gel at levels too low to detect by immunoblotting. Interaction with humic substances limiting PrP entry into the gel (4) appears likely, perhaps due to aggregation or protection from (complete) denaturation. Covalent cross-linking of PrP to humic substances (Hsu and Hatcher, 2005) cannot be excluded with the data in hand.

Studies examining the persistence in soil and compost of prions or recPrP that relied on immunoblotting for detection have typically lacked controls designed to assess signal reduction due to co-extracted NOM. Reevaluation of previous work may be necessary to confirm that loss of immunoblot signal in the presence of NOM is not erroneously attributed to PrP^{TSE} degradation. The impact of co-extracted NOM on PrP^{TSE} immunoblotting is expected to vary among studies using different soils and extractants. Studies relying on immunoblotting to detect PrP^{TSE} in experimentally manipulated environmental samples should control for potential interference using NOM specific to the samples employed. Efforts to separate proteins from humic substances are complicated by their similar chemical and physical properties and their propensity to form stable complexes. The influence of NOM on the detection of proteins from soil matrices including extracellular enzymes, Cry proteins, and glomalin warrants investigation.

Immunoblotting is expected to lack the sensitivity needed to detect the presumably low levels of prions in the environment. Nonetheless, PrP^{TSE} interaction with co-extracted NOM may impact detection by other methods including conversion assays (e.g., PMCA) and mass spectrometry. Detection of PrP^{TSE} in environmental water samples (Nichols et al., 2009) and experimentally spiked soils (Nagaoka et al., 2010; Saunders et al., 2011a; Seidel et al., 2007) by PMCA has recently been reported. The extent to which NOM affects prion detection by PMCA is unclear at present. One report found typical amplification of PrP^{TSE} spiked into ground and surface water samples with total organic carbon contents ranging between 0.34 and 7.69 mg L^{-1} (Nichols et al., 2009). Reduced PMCA efficiency was recently reported in experiments seeking to directly amplify PrP^{TSE} adsorbed to humic acid-coated SiO_2 particles (Saunders et al., 2011a); however, the influence of humic substances on the PMCA reaction was not specifically determined.

Animal bioassay remains the most definitive test for the presence of TSE infectivity in environmental samples. Measurements of prion dose typically use intracerebral inoculation, the most sensitive exposure route for TSE agents. For prion strains that have not been adapted to rodents, transgenic mice (i.e., mice in which the *Prnp* gene is ablated and that of the desired host species is inserted) can be used for intracerebral bioassays (Scott et al., 1989). Oral inoculation represents an environmentally relevant exposure route, but lacks the sensitivity needed for routine prion detection (e.g., oral transmission in hamsters is $\sim 10^9$-fold less efficient than intracerebral inoculation (Prusiner et al., 1985)). To evaluate mechanisms of environmental prion transmission, the extent that NOM impacts oral disease transmission warrants investigation.

The infectivity assay presented here indicates a statistically significant increase in the mean incubation period for PrP^{TSE} in the presence of humic acid correlating to a decrease in titer (Prusiner et al., 1982). The reason for the apparent slowing of disease progression is not clear at present but may be due to reduced bioavailability of PrP^{TSE} associated with NOM. Alternatively, humic acid may alter other processes in the brain in a manner that impacts prion propagation. These bioassay data demonstrate that prions mixed with humic acid remain infectious to animals despite reduction in or even undetectable PrP^{TSE} immunoblot signal. Taken together, our immunoblot and animal challenge data indicate that studies of PrP^{TSE} interactions with soils or humic acids should corroborate reductions in PrP immunosignals with other measures of PrP^{TSE} activity, such as PMCA or bioassays.

Attachment of prions to montmorillonite particles was previously shown to slightly enhance disease progression in hamsters exposed intracerebrally (Johnson et al., 2006). Montmorillonite and NOM appear to have opposing effects on the incubation period in intracerebrally dosed hamsters. While soil particles and humic substances clearly impact accurate measurement of disease titers in environmental samples, intracerebral bioassays remain a viable method to demonstrate the presence of TSE infectivity and provide an estimate of effective titer. Our data and those of Johnson et al. (2006) suggest that the effective titer for NOM- or mineral particle-associated prions is within ~1-log of that of prions in the absence of soil constituents. Using serial dilutions, Saunders et al. (2011a) measured a 1.3-log reduction in the titer of HY agent upon binding to silty clay loam soil, which confirmed an inverse correlation between incubation period and prion dose, for prions associated with soil particles.

To date, studies focusing on the interaction of PrP^{TSE} with NOM have not appeared in the literature. NOM or NOM surrogates appear to enhance α-recPrP binding to soil particles (Polano et al., 2008; Pucci et al., 2008; Rao et al., 2007). While recent studies have examined enzymatic digestion (Saunders et al., 2010; Saunders et al., 2011b) and *in vitro* replication (Saunders et al., 2011c) of *bona fide* PrP^{TSE} bound to humic acid-coated SiO_2 microparticles (Saunders et al., 2010, 2011b), the impact of humic acid on PrP^{TSE} attachment and detection remains unclear. Potential mechanisms of NOM interference on PrP^{TSE} immunoblotting appear to involve direct interactions between PrP^{TSE} and NOM. Co-precipitation of PrP^{TSE} and ESHA by methanol and NaPTA provide additional evidence of protein-humic association. Our data suggest that PrP^{TSE} interacts with at least some NOM components (e.g., polyphenolic structures) and that NOM can modulate prion disease progression (at least by the intracerebral route of exposure). Complexation with humic substances has been suggested to enhance protein persistence in soils (Hsu and Hatcher, 2005; Zang et al., 2000), and may contribute to the preservation of prions in soil environments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.10.005.

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Supplementary Data for

Humic substances interfere with detection of pathogenic prion protein

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Table 1. Selected physicochemical properties of the humic substances

Humic Substance	IHSS Product Code	Polarity Index (O+N+S)/C	Carbon Distribution[a]		Acidic Group Site Densities[b]	
			Aromatic	Aliphatic	Q_1	Q_2
ESHA	1S102H	0.67	50	16	8.90	0.85
LHA	1S104H	0.52	58	14	8.17	1.13
SRHA	2S101H	0.83	31	29	10.69	2.28
PPHA	1S103H	0.75	47	19	9.64	0.94
ESFA	1S102F	0.93	30	22	ND	ND

[a] Relative carbon distribution estimated from ^{13}C NMR spectra and reported as percentages of integrated peak area for the 165-110 ppm and 60-0 ppm chemical shift ranges assigned as aromatic and aliphatic carbon, respectively (Thorn et al., 1989). [b] Acidic group site densities, Q_1 and Q_2, are fitting parameters describing the site densities ($meq \cdot g \cdot C^{-1}$) of (predominately) carboxylic and phenolic functional groups, respectively (Ritchie and Perdue, 2003).

Table 2. Physicochemical properties of the soil and compost samples[a]

Parameter	Elliot Soil	Pahokee Peat Soil	Compost
Total N[b]	0.0025	0.031	0.011
C/N ratio[b]	11.6	14.6	8.57
Specific surface area[c] ($m^2 \cdot g^{-1}$)	10	1.8	1.8
f_{oc}[d]	0.029	0.457	0.085

[a] Abbreviations: f_{oc}, mass fraction of organic carbon. [b]Elemental analyses of bulk soil samples provided by IHSS. [c] Specific surface area measured by N_2 adsorption (BET method). [d] Mass fraction of organic carbon determined by the organic carbon dry combustion method using a Leco CNS-2000 (St. Joseph, MI).

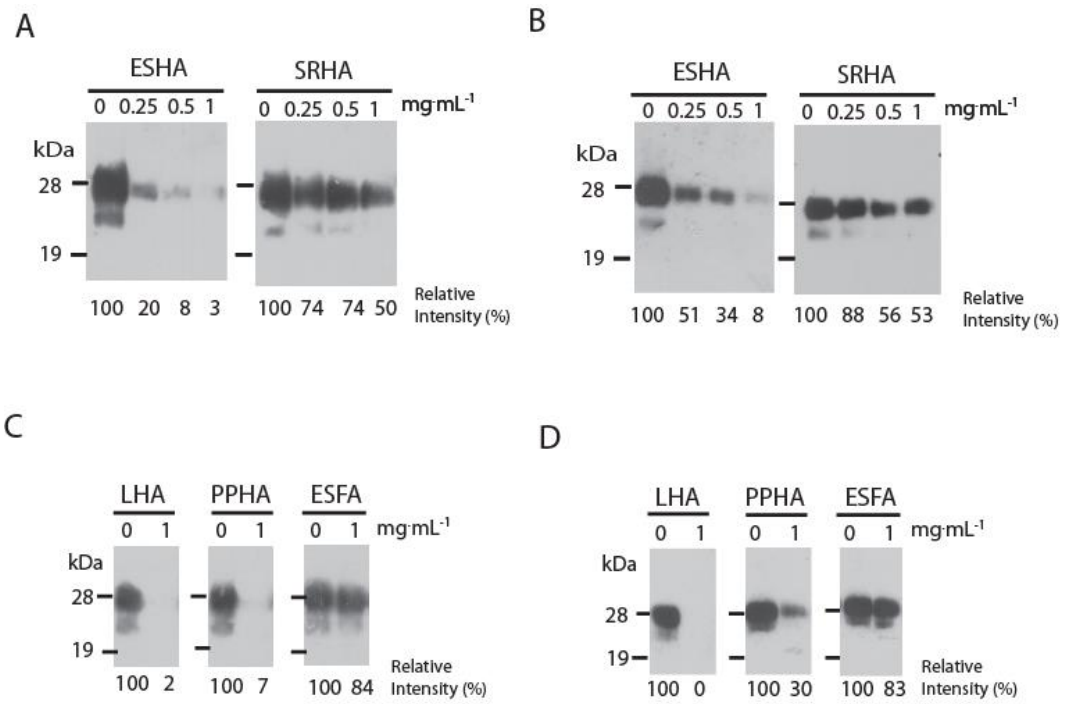
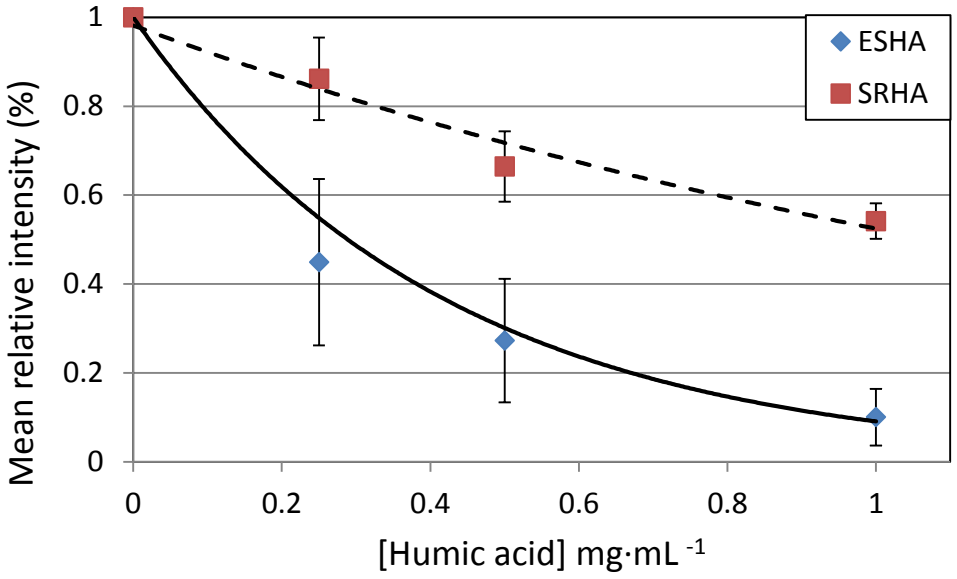


Figure 1. The impact of humic substances on PrP^{TSE} immunoblots is consistent in replicate experiments. Purified PrP^{TSE} (0.1 µg) was incubated (1 h) with increasing amounts of ESHA and SRHA (A and B) or LHA, PPHA, and ESFA (1 mg·mL^{-1}) (C and D). The reduction in PrP^{TSE} levels was estimated by densitometry. Immunoblots were probed with mAb 3F4.

A



B

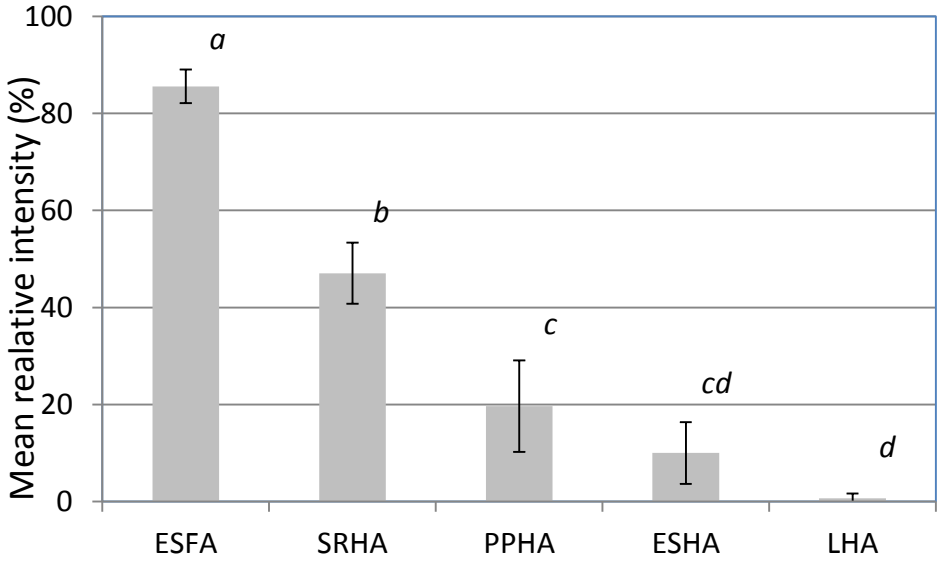


Figure 2. The degree of immunoblot inhibition increases with decreasing humic substance polarity. (a) Purified PrP^{TSE} (0.1 µg) was incubated (1 h) with increasing amounts of Elliot soil humic acid (ESHA) or Suwannee River humic acid (SRHA). (b) Impact of humic substance type on PrP^{TSE} immunoblotting. PrP^{TSE} (0.1 µg) was incubated (1 h) with 1 mg·mL^{-1} of each humic substance as indicated (ESFA, Elliot soil fulvic acid; PPHA, Pahokee peat humic acid; LHA, Leonardite humic acid). Experiments were conducted in triplicate, and the reduction in PrP^{TSE} levels was estimated as the mean percent relative intensity measured by densitometry. Error bars represent the standard deviation from the mean. The extent of immunoblot interference at varying concentrations of humic acid differed for ESHA and SRHA (ANOVA, $p < 0.05$). Based on statistical comparison of the five humic substances, mean relative intensities labeled with the same letter are not significantly different ($p > 0.05$). Immunoblots were probed with mAb 3F4.

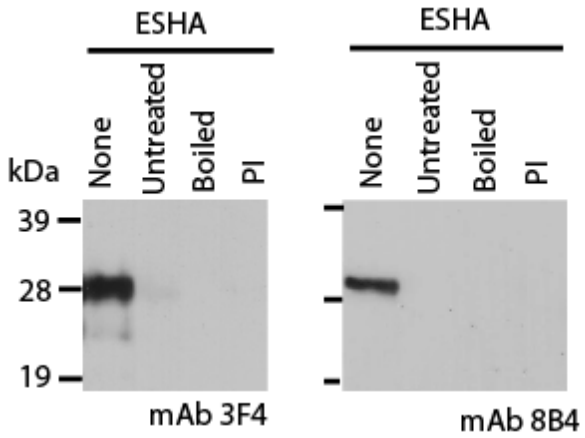


Figure 3. Treatments to eliminate protease activity in ESHA did not restore PrP^{TSE} immunoblot signal. ESHA (1 $mg \cdot mL^{-1}$) was boiled for 10 min or incubated with Complete protease inhibitor cocktail tablets EDTA-free (Roche Applied Science, Mannheim, Germany) at 12× concentration (PI) before incubating with purified PrP^{TSE}.

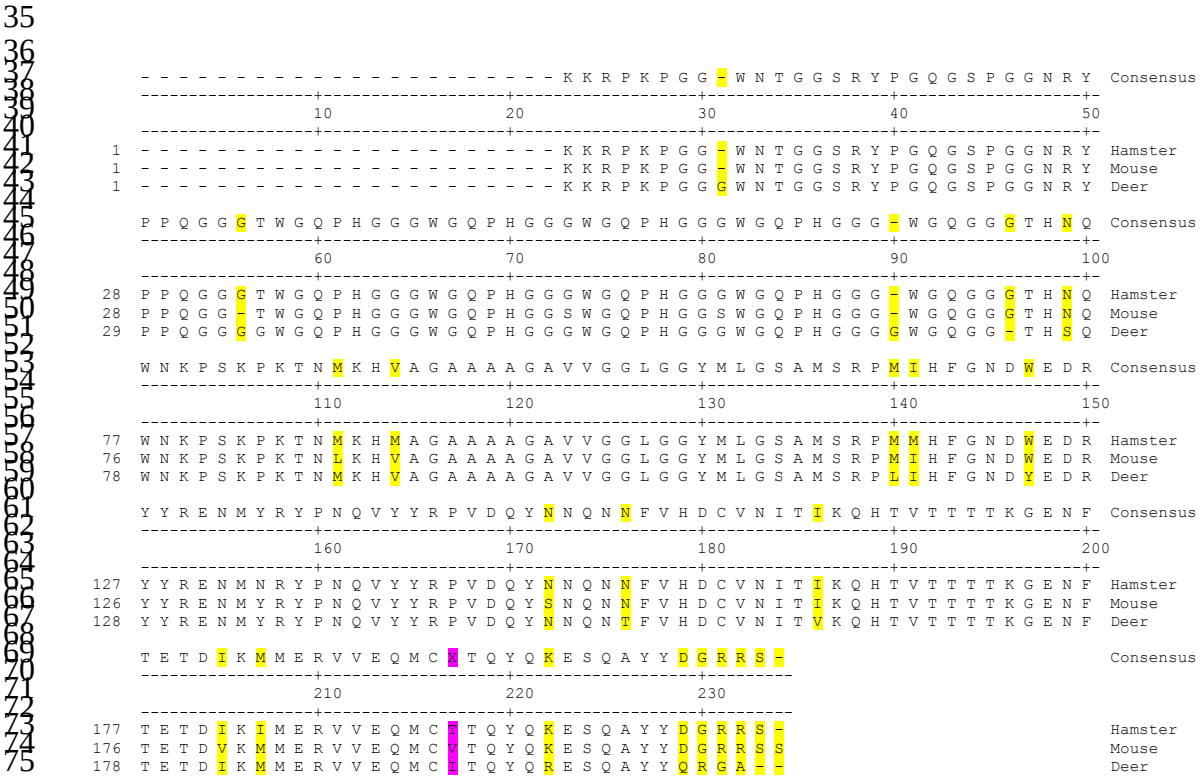


Figure 4. The amino acid sequences for the mature prion protein of white-tailed deer (residues 25-233), Syrian hamster (residues 23-231) and mouse (residues 23-231). Sequences were aligned by MegAlign software (DNASTAR, Madison, WI) using Clustal W analysis. Numbering is based on the consensus sequence. Positions in the consensus sequence that exhibit one difference are highlighted in yellow. Residue 217, which exhibits no agreement between the three species, is designated X and highlighted pink.

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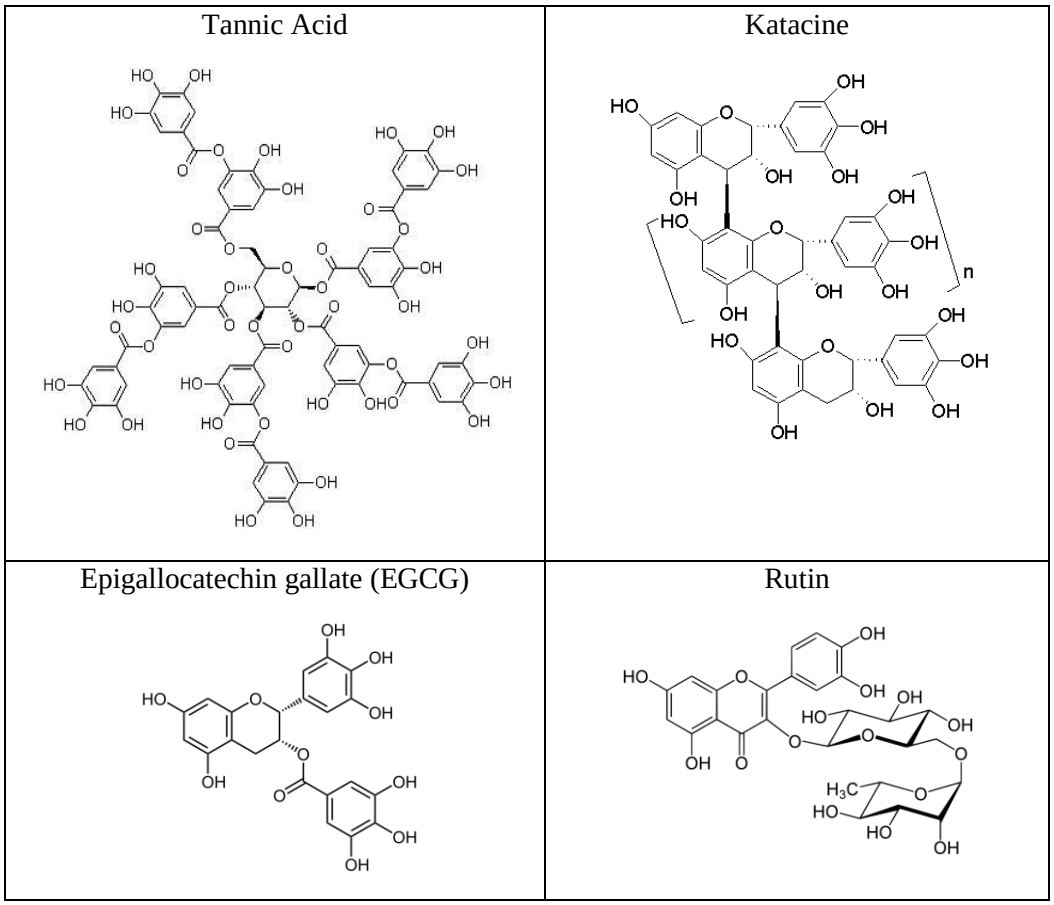

85
86 **Figure 5. Molecular structures of tannic acid, katacine, epigallocatechin gallate (EGCG),**
87 **and rutin.**

Incubation time interval assay. Our estimation of infectious titer using the incubation time interval assay (Prusiner et al., 1982) relies on several assumptions. First, the relationship between titer and incubation period was developed for the 263K strain of hamster-adapted scrapie agent. The 263K and HY strains represent different hamster-adapted TSE strains because of their different disease origins (scrapie vs. transmissible mink encephalopathy); however, the PrP^{TSE} associated with these strains have very similar biochemical and physical properties (Bessen and Marsh, 1992). Extension of the incubation time interval assay to HY agent assumes that it exhibits a relationship between titer and incubation period similar to that of 263K. Second, we have applied the incubation time interval assay to a partially purified preparation of PrP^{TSE}. The log-linear relationship between titer and incubation time was determined using dilutions of 10% infected brain homogenate. Partial purification increases the average aggregate size of PrP^{TSE}, which may result in slight deviation from the linear relationship between the logarithms of dilution and dose. In light of these assumptions, the estimates of the reduction of disease titer in the presence of ESHA or SRHA should be considered semi-quantitative. Extension of our results to different prion concentrations or other humic substances may be limited.

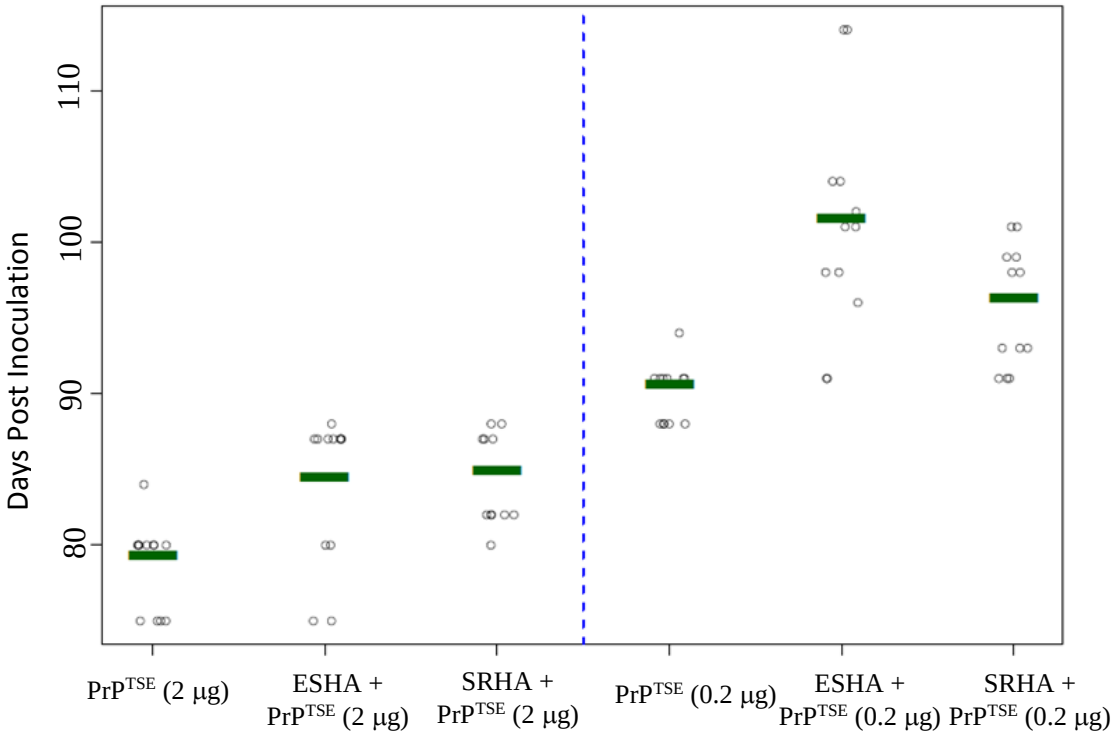

Figure 6. Time period required for the onset of clinical signs for hamsters receiving the indicated inocula. Geometric means for each cohort are marked (green line).

Table 3. Estimated multipliers from the log-linear model of time to clinical signs

Exposure	Estimated multiplier (95% CI)	*p* value
ESHA vs. control	1.093 (1.063, 1.124)	< 0.0001
SRHA vs. control	1.066 (1.037, 1.097)	< 0.0001
High vs. low dose	0.861 (0.841, 0.881)	< 0.0001

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