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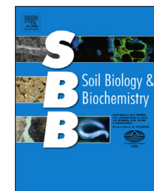

Humic substances interfere with detection of pathogenic prion protein

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A B S T R A C T

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.

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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

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