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A diagnostic test for scrapie-infected sheep using a capillary electrophoresis immunoassay with fluorescent-labeled peptides

Scrapie in sheep and goats is the prototype of transmissible spongiform encephalopathies found in humans and animals. A feature of these diseases is the accumulation of rod-shaped fibrils in the brain that form from an aggregated protein. This protein (PrP^{SC}) is a protease-resistant form of a normal host cell protein. When the aggregated protein is denatured in sodium dodecyl sulfate (SDS) and β-mercaptoethanol, a monomer form of ~ 27 kDa molecular mass is observed. A competition immunoassay to detect PrP^{SC} from scrapie-infected sheep was developed using free zone capillary electrophoresis with laser-induced fluorescence (LIF) for detection and flourescein-labeled synthetic peptides from PrP^{SC}. Antibodies were made to each respective peptide and used in the competition assay. The fluorescent-labeled peptides bound to the antibody were separated from the unbound peptides using 200 mM Tricine, pH 8.0, containing 0.1% *n*-octylglucoside and 0.1% bovine serum albumin (BSA). The amount of antibody that would bind ~50% of the fluorescent-labeled peptide was determined for each peptide. When unlabeled peptide was added to the assay, ~2 fmoles of the peptide could be measured. When PrP^{SC} extracted from infected sheep brain was added to the assay, approximately 135 pg of PrP^{SC} could be detected. When preparations from normal sheep were assayed, there was little or no competition for the bound peptides. Assays using two of the peptides, peptides spanning amino acid positions 142–154 and 155–178, clearly differentiated scrapie-positive sheep from normal animals. This assay is a new method that can be used to diagnose scrapie and, possibly, other transmissible spongiform encephalopathies in animals and in humans.

1 Introduction

Transmissible spongiform encephalopathies (TSEs) cause progressive degenerative disorders of the central nervous system resulting in death [1]. Because there is no known treatment for these diseases, early detection of the disease before clinical signs appear could lead to better control of the disease. Although scrapie, a TSE in sheep and goats, has been described and known in sheep for over 200 years [2], it is still diagnosed by postmortem examination of the tissues of the animals. Because of the recent outbreak of bovine spongiform encephalopathy in the United Kingdom [3] and the possible connection between this TSE and the new variant Creutzfeld Jacob [4], a human TSE, there is a need to develop a test for TSEs that is sensitive and accurate. Ideally, this test could be used to test animals before they show clinical signs and before they enter the human food chain or into pharmaceuticals prepared for human use.

Most of the data suggest that scrapie and other TSEs are caused by an alteration of a host glycoprotein through a post-translational *N*-terminal truncation. This post-translational modification causes a conformational change in the protein [5, 6] that causes the protein to aggregate and to become resistant to proteases. The properties of the altered host glycoprotein (PrP^{SC}) are used to partially purify the aggregated protein from infected brains. Tests for PrP^{SC} include immunohistochemistry [7, 8], immunoblot [9–11], and an ELISA test [12]. Antiserum made to synthetic peptides of the prion protein react with the dissociated aggregates on Western blot analysis [13]. Using this information and recent reports on the use of capillary electrophoresis (CE) for detection of proteins of biological interest [14–19], we studied the possibility of using CE with laser-induced fluorescence detection and immune complex formation as a method to detect scrapie in sheep. We used three approaches to the problem. In the first study [20], we used direct binding of the peptide antiserum to the scrapie prion protein and used fluorescent-labeled antibody to rabbit antibody for detection of the immune complexes. Although the scrapie prion protein was detected by this method, the broad peak shapes made it difficult to obtain accurate measurements and reproducibility was a problem. In the second approach [21, 22], a fluorescent-labeled peptide and whole rabbit antiserum was used. The sensitivity was increased by this competition assay but reproducibility was still a problem due to incomplete removal of the denaturants used to prepare the samples. In a third approach, reported here, we used IgG fractions of rabbit antiserums and a 200 mM Tricine, pH 8.0, buffer containing 0.1% *n*-octylglucoside and 0.1% BSA and an improved method of purification for processing the samples. Using this method, we were able to diagnose scrapie-infected sheep accurately and reproducibly.

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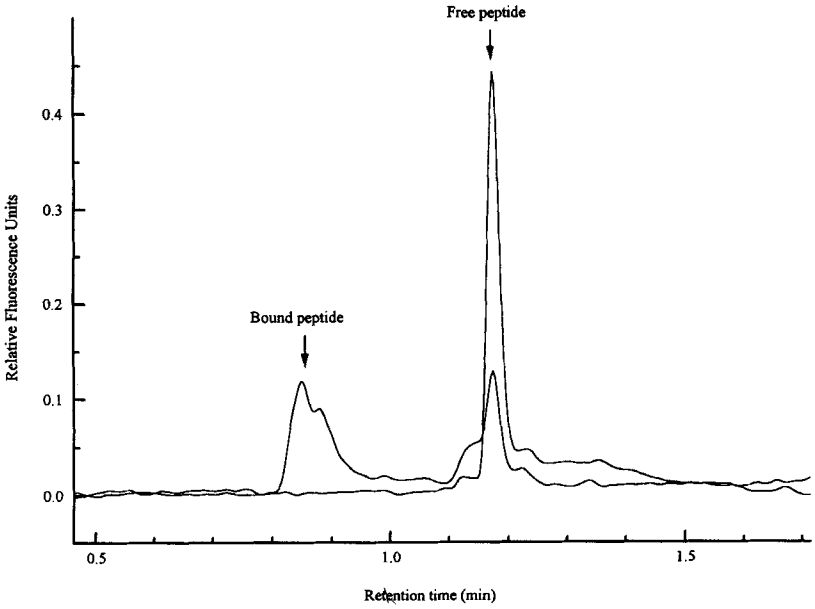

Figure 1. Electropherograms showing the fluorescent-labeled peptide 142–154 alone and immunocomplex formation in the presence of rabbit IgG.

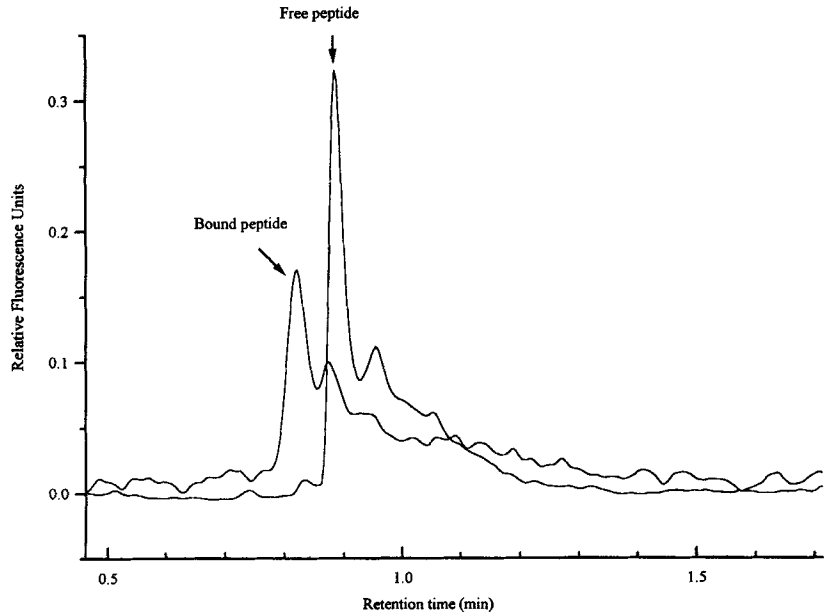

Figure 2. Electropherograms showing the fluorescent-labeled peptide 155–177 alone and immunocomplex formation in the presence of rabbit IgG.

2 Materials and methods

2.1 Preparation of sheep brain material

Scrapie-infected brains were obtained from sheep that were confirmed positive by histological examination, immunohistochemistry [7] and by Western blot [11]. Normal brains were obtained from sheep from a scrapie-free flock and were confirmed negative by the above tests. All of the brain material was prepared by a modification of Bolton *et al.* [23]. Briefly, the brain stem was dissected from the whole brain, weighed, placed in 0.32 M sucrose (10% w/v), and homogenized for 60 s with a Brinkman Polytron (Kinematica AG, Lucerne Switzerland) using a 0.7 cm stainless steel generator at the highest speed. (Following the procedure, the generator was decontaminated in 5.25% sodium hypochlorite.) The homogenate was centrifuged at 10 000 *g* for 20 min to remove particulates, and the resultant supernatant fluid was centrifuged at 230 000 *g* for 1 h. The pellet was resuspended in 20 mM Tris pH 7.4, 0.15 M NaCl and centrifuged at 230 000 *g* for 1 h. This pellet was resuspended in 10 mM Tris, pH 7.4, containing 10% sodium *N*-lauroyl sarcosine and centrifuged at 230 000 *g* for 1 h. The pellet was resuspended in 10 mM Tris, pH 7.4, and incubated with proteinase K (10 μg/mL) for 1 h at 37°C and held overnight at 4°C and then centrifuged at 230 000 *g* for 1 h. The final pellet was resuspended in 10 mM Tris, pH 7.4 (100 μL/g of the initial brain sample). The sample was solubilized in 0.01 M Tris-HCl, pH 8.00, containing 2 mM EDTA, 5% SDS and 10% hexafluoroisopropanol at 100°C for 10 min and applied to PolyWAX LP (Poly LC, Inc., Columbia, MD) high performance liquid chromatography column (200 × 4.6 mm) in 100%

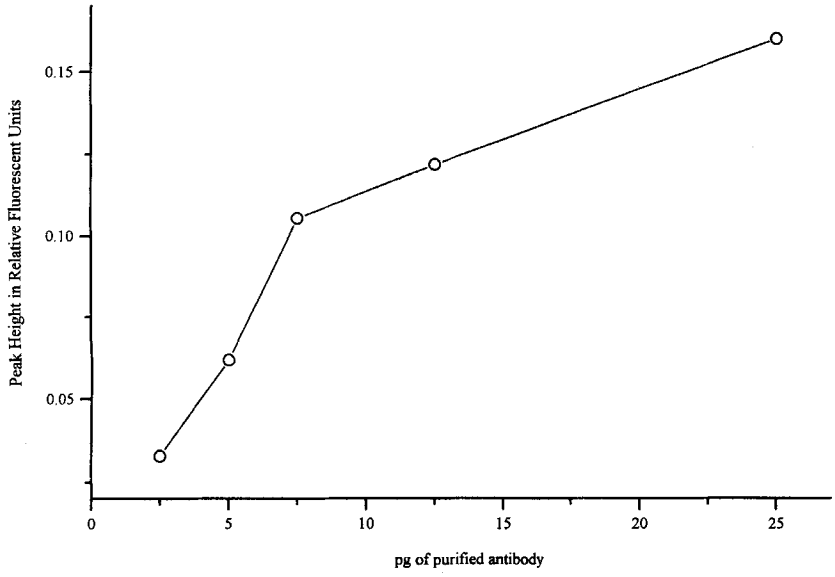

Figure 3. Plot of the antibody concentration *vs.* amount of the bound (represented by peak height of the immunocomplex) for peptide 142–154.

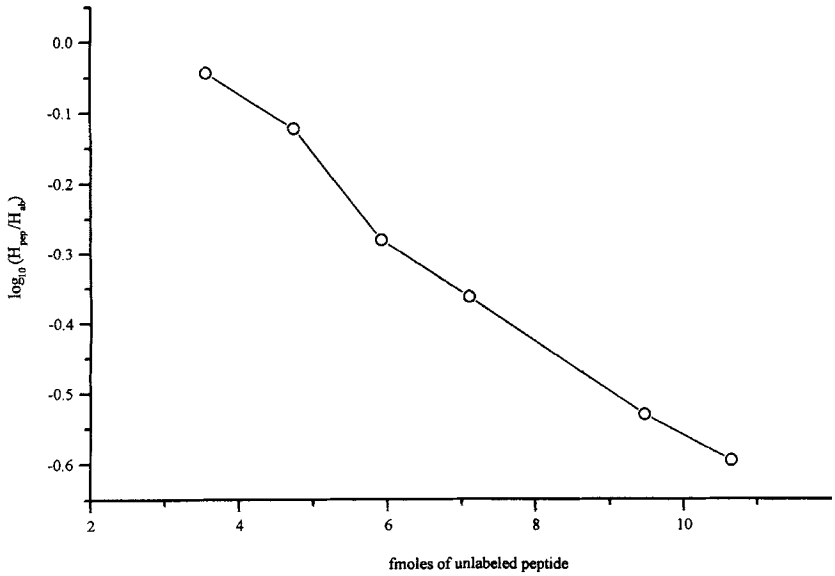

Figure 4. Plot of the unlabeled peptide (142–154) concentration *vs.* $\log_{10}$ (peak height of peptide/ peak height of antibody control).

acetonitrile containing 0.1% TFA acid and 50 mM hexafluoroisopropanol (buffer A). The flow rate was 0.5 mL/min. The conditions for eluting PrP^{SC} were 100% A for 8 min and then a linear gradient to 100% water containing 0.1% TFA and 50 mM hexafluoroisopropanol (buffer B) in 15 min, 100% B for 10 min. Peak fractions were collected and dried in a vacuum centrifuge (Savant Instruments, Inc., Farmingdale, NY). Fractions were resuspended in 10 μL of double-distilled water and the fraction that tested positive by immunoblot for PrP^{SC} was used in the capillary electrophoresis assay.

2.2 Preparation of peptides and antibodies

Four peptides from the prion protein were synthesized (Multiple Peptide Systems, San Diego, CA). The peptide sequences were CGQGGGTHNQYNKPSK (spanning amino acid positions 89–103), CKTNMKHVAGAA-AAGAVVGGLG (106–126), CNDWEDRYYRENMYR (142–154), and CRYPNQVVYRPVDRYSNQNNFVHD (155–178). The peptides were labeled with fluorescein through a γ-butyric acid linkage on the *N*-terminus during synthesis. Rabbits were immunized with each peptide and specific antibodies were produced for each peptide (Multiple Peptide Systems). These antiserums reacted with scrapie-infected brain but not with normal brain on Western blot analysis [11]. Rabbit IgG was prepared by passing each antiserum over a protein G HiTrap affinity column (Pharmacia Biotech, Uppsala, Sweden) according to the directions of the manufacturer. A ProtOn™ Kit (Multiple Peptide Systems) was used to produce affinity purified antibodies. Briefly, 10 mg of a peptide was coupled to agarose resin modified with an *N*-hydroxylsuccinimide ester in 1.0 mL dimethylformamide at room temperature for 20 min. After coupling, the resin was washed with 5 mL of 0.1 M MOPS, pH 7.5 (column wash buffer). Unreacted ester groups were deactivated with 0.1 M HEPES, pH 8.0, and 0.1 M NH_4Cl for 15 min. Before antibodies were applied to the peptide columns they were purified using protein G chromatography. After diluting 1:2 in column wash buffer, the

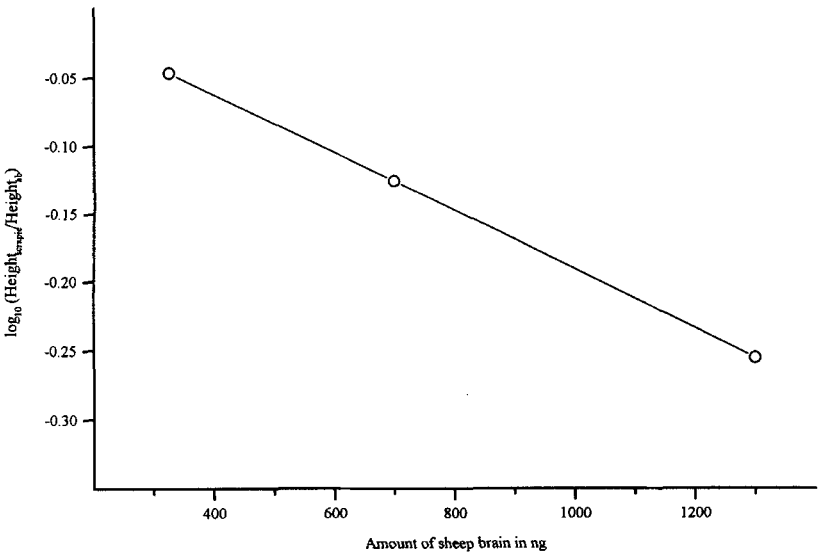

Figure 5. Plot of the amount of calculated brain sample containing PrP^{SC} *vs.*/$\log_{10}$ (peak height of brain sample/peak height of antibody control).

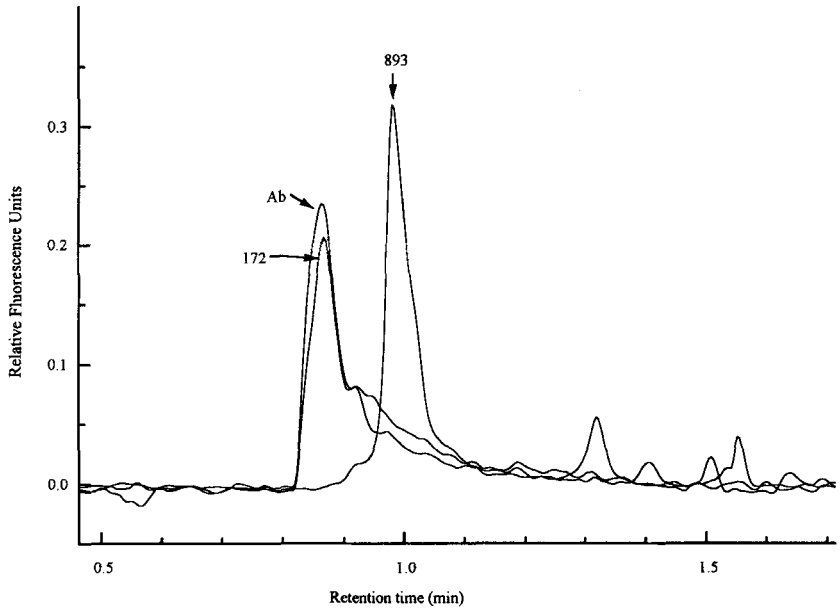

Figure 6. Electropherograms showing immunocomplex formation in the presence of rabbit IgG using fluorescent-labeled peptide 155–177, immunocomplex formation in the presence of a normal sheep brain preparation (172) and in the presence of a scrapie-infected sheep brain (893).

antibodies were applied to the affinity column and recycled over the column for several cycles. The column was then washed with column wash buffer and the antibodies eluted with 0.1 M NaH_2PO_4, pH 2.5, into tubes containing 50 µL of 1 M HEPES, pH 8.5. The absorbance was measured at 280 nm. Fractions with abs_{280nm} were pooled, aliquoted and frozen at –20°C.

2.3 Immune complex formation

Fifteen microliters of fluorescein-labeled peptide containing ~20 pmoles of the fluorescent-labeled peptide were mixed with varying amounts (0.5–5 µg) of purified rabbit IgG to determine the antibody concentration that forms ~50% of the total immune complex formation. The final volume of the sample was adjusted to 20 µL with capillary running buffer. After mixing the components, the samples were incubated at 25°C for ~10 min. The peak height of the immune complex peak was measured for duplicate samples for each calculation. Replicates of samples varied less than 1%. Known amounts of unlabeled peptides corresponding to the fluorescent-labeled peptides were used to generate standard curves. Dilutions of preparations from pooled sheep brain were assayed. One µL samples were used to test individual animals.

2.4 Capillary electrophoresis conditions

Free-zone capillary electrophoresis was performed on a Beckman P/ACE 5500 (Beckman Instruments, Fullerton, CA) controlled by P/ACE Station software (Beckman Instruments). LIF detection was done using an air-cooled argon laser (Beckman Instruments) with excitation at 488 nm and emission at 520 nm. Unmodified capillaries were obtained from Beckman Instruments. A

20 cm (length to the detector) × 20 μm ID capillary was used with a 200 mM Tricine buffer that was adjusted to pH 8.0 by 6 N NaOH. This buffer contained 0.1% *n*-octylglucoside (Boehringer Mannheim GmbH, Indianapolis, IN) and 0.1% BSA (Sigma Chemical Co., St. Louis, MO). In preparation for the separation, the capillary was rinsed for 1 min with 0.25 M NaOH, rinsed for 2 min with H_2O, and then rinsed 2 min with buffer. The separating conditions were 30 kV for 3 min at 20°C. The current was ~20 μA. The sample was injected for 15 s followed by a 5 s injection of running buffer. The sample volume was ~ 0.95 nL. Rinses were carried out under high pressure and sample injection was carried out under low pressure.

3 Results and discussion

In this study, sensitivity and reproducibility were improved considerably over the previously reported assay system [22]. The inclusion of 0.1% *n*-octylglucoside improved the solubility of the PrP^{SC} and the inclusion of 0.1% BSA prevented PrP^{SC} from binding to the capillary walls. The sensitivity of the assay was enhanced 100-fold relative to the previously reported procedure [22] by the addition of fluorescein at the amino terminal during synthesis. The additional purification of PrP^{SC} with hydrophilic interaction chromatography improved the reproducibility of the assay dramatically. As previously reported [22], immunocomplex peaks were observed for peptides spanning the amino acid positions 89–103 and 142–154. In this study, we repeated the assay for peptide 142–154 (Fig. 1) and studied the peptides 106–126 and 155–177 which had not been reported previously. A result similar to that of the first two peptides was observed for peptide 155–177 (Fig. 2). When antibody was added to the fluorescent-labeled peptide spanning amino acid positions 106–126, quenching of the fluorescence was observed. Although the quenching was dependent upon the concentration of the antibody, the calculations were more complicated and, as a result, we did not pursue the use of this peptide for an assay for the scrapie-infected sheep. The migration distance between the immunocomplex peak and the free peptide for peptide 142–154 was 0.31 min and for peptide 155–177 it was 0.064 min. A plot of anti-peptide 142–154 antibody concentration *vs.* the amount of immunocomplex formed is shown in Fig. 3. A similar curve (not shown) was obtained for peptide 155–177. The concentration of antibody was chosen so that the antibody would be limiting in the assay. The amount of peptide 142–154 was 4.0 fmoles in the capillary, and of peptide 155–177, 7.5 fmoles. The antibody for peptide 142–154 was purified over protein G affinity chromatography. The antibody to peptide 155–177 was affinity purified. The competition of unlabeled peptide (142–154) with fluorescein-labeled peptide was concentration dependent (Fig. 4). A similar plot was obtained for peptide 155–177 (not shown). A plot was made with varying concentrations of PrP^{SC} to determine if there would be a different relationship with the whole protein compared to the peptide competition. As can be seen in Fig. 5, there was a concentration dependence on PrP^{SC} for competition with the labeled peptide. Electropherograms representing the immunocomplex peak, a preparation from a normal sheep and a preparation from a scrapie-infected sheep are shown in Fig. 6 (peptide 155–177). When ratios of peaks from samples from normal sheep to the control immunocomplex peak were made, the ratios ranged from 0.90 to greater than 1.0, indicating little or no reduction in the immunocomplex peak. The scrapie-infected sheep often had no immunocomplex or had peak ratios less than 0.5 (Table 1). For all of the sheep tested, this assay was diagnostic for the scrapie-infected sheep.

Table 1. The results of the individual sheep tested by the capillary electrophoresis method

Sheep number	Scrapie status[a)]	Peptide 2 PkH_{sheep}/PkH_{ab}[b)]	Peptide 4 PkH_{sheep}/PkH_{ab}[b)]
22	–	1.06	0.951
172	–	0.88	1.22
215	–	0.922	N.D.[c]
893	+	0.108	0.012
807	+	0.407	N.D.[c)]
W95	+	0.493	N.D.[c)]
SBT (pool)	+	0.033	0.012
SBT1,2,3 (pool)	+	0.068	0.012

a) Tested by immunohistochemistry and immunoblot
b) Ratios below 0.85 were positive for scrapie infection
c) Not tested

4 Concluding remarks

The capillary electrophoresis assay described in this study is reproducible, more sensitive and faster than the previous tests. Although the samples tested in the capillary electrophoresis assay were obtained from brains postmortem, the sensitivity of this assay makes it possible to test samples from other tissues that contain much less PrP^{SC} than brain samples. This may make it possible to examine tissues such as blood that are available from live animals and diagnose animals prior to the onset of clinical signs. Because of the inherent limitations of the other diagnostic tests such as immunohistochemistry [7, 8], ELISA [12], and immunoblotting techniques [9–11], antemortem assays have not been a possibility. In addition to testing for TSE infection in animals, this test could be used for process material prepared from animals for human use. The possibility for automation could lead to more economical and efficient methods for testing for the presence of PrP^{SC}, the marker for infection with a transmissible spongiform encephalopathy.

No endorsements are herein implied. Brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standards of the products, and the use of the names by the USDA implies no approval of the products to the exclusion of others that may also be suitable.

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