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Improvements in a competition assay to detect scrapie prion protein by capillary electrophoresis

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Abstract

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 is a protease-resistant form of a normal host cell protein. When the aggregated protein is denatured in SDS and β-mercaptoethanol, a monomer form (prion protein) with a molecular mass of 27 kDa is observed. Free zone capillary electrophoresis and peptides labeled with fluorescein were used to detect the prion protein through competition for a labeled peptide in immune complex formation. The separation of the immune complexes from the unbound peptide using 200 m*M* Tricine (pH 8.0) was faster and was better resolved than that obtained with phosphate or borate buffer systems. The amount of immune complex formation was dependent on the amount of antibody in the assay. The amount of bound labeled peptide and unbound labeled peptide could be measured directly by calculating the area of each respective peak. As increasing amounts of unlabeled peptide were added to the assay, a concentration dependent reduction in the immune complex peak was observed. The assay could detect less than 10.0 fmol of unlabeled peptide. There was a quantitative difference in the competition of preparations from scrapie infected sheep brain and normal sheep brain.

Keywords: Proteins

1. Introduction

Scrapie of sheep and goats is the prototype of other transmissible spongiform encephalopathies (TSE) found in humans and in animals [1]. These TSE cause progressive degenerative disorders of the central nervous system resulting in death. Because there is no known treatment for these diseases, detection of the presence of the disease causing agent early in infection before clinical signs appear could lead to the better control of the disease. Although scrapie has been described and known in sheep for 200 years [2] and TSE are relatively rare in other species including humans, concern has been increasing recently because of the diagnosis of TSE in cattle in the British Isles [3]. The cause of this infection has been attributed to the feeding of bone meal contaminated with the disease agent [4].

Unlike other infectious diseases that are caused by viruses, bacteria, parasites or fungi, TSE are caused by an accumulation in the lysosomes of a modified normal cellular protein [5,6]. This protein is modified

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by a post translational modification which truncates a host cellular protein at the N-terminus [7] causing a conformational change in the protein [1,8]. After modification, this protein becomes resistant to proteases and aggregates into rod-shaped fibrils in the brains of infected animals. These properties are used to partially purify the fibrils from infected brains [9]. When the aggregates are subjected to SDS-PAGE in the presence of β-mercaptoethanol, a monomer form (prion protein) is observed with a molecular mass of ~27 kDa [10]. Antiserum made to synthetic peptides from this monomer protein (prion protein) will react with the dissociated aggregates on Western blot analysis [11]. Using this information and recent reports on the use of capillary electrophoresis (CE) for detection of proteins of biological interest [12–16], 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 [17], we used direct binding of the peptide antiserum to the scrapie prion protein. A fluorescent labeled antibody to rabbit antibody was used to detect the immune complexes. Although the scrapie prion protein was detected by this method, the broad peak shapes made it difficult to do accurate measurements. In the second approach [18], a fluorescent labeled peptide and whole rabbit antiserum was used. A borate buffer system was used in this assay. The sensitivity was increased by this competition assay but again the peaks were broad and unsymmetrical. In the third approach, that is reported here, we used IgG fractions of the rabbit antiserums and a 200 m*M* Tricine (pH 8.0) buffer. The peak shape was improved and accurate measurements were obtained.

2. Experimental

2.1. Preparation of sheep brain material

A scrapie-infected brain, confirmed positive by histological examination and by immunohistochemistry [19], was obtained from a sheep. Normal brain was obtained from a sheep from a scrapie-free flock and confirmed negative by the above tests. The brain material was prepared by a modification of the method of Prusiner et al. [9]. 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. The homogenate was centrifuged at 10 000 *g* to remove particulates, and the resultant supernatant fluid was centrifuged at 230 000 *g* for 1 h. The pellet was resuspended in 20 m*M* Tris (pH 7.4)–0.15 *M* NaCl and centrifuged at 230 000 *g* for 1 h. This pellet was resuspended in 10 m*M* Tris (pH 7.4)–10% sodium N-lauroyl sarcosine and centrifuged at 230 000 *g* for 1 h. The pellet was resuspended in 10 m*M* 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 m*M* Tris (pH 7.4) (100 μl/g of the initial brain sample).

2.2. Peptide synthesis, antibody production and fluorescein labeling

Peptides spanning amino acids 89–103 (pep 1) and 142–154 (pep 2) of the scrapie prion protein were synthesized (Chiron Mimotopes Peptide Systems, San Diego, CA, USA) with an attached cysteine at the N-terminus. Rabbit antiserum was produced to each of the peptides (Chiron Mimotopes Peptide Systems). These antiserums reacted with scrapie-infected brain, but not with normal brain, on Western blot analysis [17]. Rabbit IgG was prepared by passing the antiserum over a protein G HiTrap affinity column (Pharmacia Biotech, Uppsala, Sweden) according to the directions of the manufacturer.

The method described by Brinkley [20] was used for labeling the peptides with fluorescein. Briefly, fluorescein iodoacetamide (Fl-I) (Molecular Probes, Eugene, OR, USA) was dissolved in dimethyl formamide (1 mg/0.1 ml) and mixed with the peptide in 20 m*M* sodium phosphate (pH 7.2) at a molar ratio of 1 part Fl-I to 1 part peptide for 2 h in the dark at 25°C. The reaction vessel was purged with nitrogen gas during the reaction. The coupling reaction was stopped by the addition of 0.1 *M* dithiothreitol. The sample was placed over a P-4 gel (Bio-Rad, Hercules, CA, USA) to separate the labeled peptide from the unreacted label. The absorbance of the column eluate was monitored at 280 nm and 488 nm. Peak

tubes containing the labeled peptide were pooled, aliquoted and frozen at −20°C in the dark. The labeling efficiency of the peptide was calculated by measuring the amount of peptide in the labeled peptide by Lowry et al. [21] and by competition radioimmunoassay [22]. The amount of fluorescein incorporated into the labeled peptide was measured by reading the absorbance at 491 nm and using the molar absorptivity of fluorescein to calculate the concentration of the fluorescein.

2.3. Immune complex formation

Fluorescein-labeled peptide (10 μl; ~3.76 μM for pep1 and 62.0 μM for pep2) was mixed with varying amounts of purified rabbit IgG (5.0–0.5 μl) to determine the antibody concentration that forms ~50% of the total immune complex formation. The final volume of the sample was adjusted to 30 μl with capillary running buffer. After mixing, the components the samples were incubated at 25°C for ~10 min. The areas of the immune complex peak and of the free labeled peptide peak were calculated using the system Gold software (Beckman Instruments) that was used in conjunction with the P/ACE. The ratio of the bound labeled peptide vs. the free labeled peptide was determined by dividing the area of the immune complex peak by the area of the free labeled peptide peak. Duplicate samples were done for each calculation. Variation from sample to sample was 5–8%. The prepared brain material from either scrapie-infected or normal brain was diluted so 1:2 in 20 mM Tris (pH 8.0) containing 2 mM EDTA, 5.0% SDS and 5% β-mercaptoethanol, and boiled for 10 min. After denaturation, the protein was diluted in capillary running buffer 1:50, 1:75, 1:100 and 1:200. The 1:50 dilution was set to equal one brain equivalent. Of each dilution 10 μl was used in the assay. Samples to produce a standard curve for competition with the unlabeled peptide were included in the assays for prion protein in brain samples.

2.4. Capillary electrophoresis conditions

Free zone capillary electrophoresis was performed on a Beckman P/ACE 5500 (Beckman Instruments) controlled by System Gold software (Beckman Instruments). Laser-induced fluorescence detection was done using an air-cooled argon laser (Beckman Instruments) 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 I.D. capillary was used with a 200 mM Tricine buffer that was adjusted to pH 8.0 with 6 M NaOH. 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 4 min at 20°C. The current was 38.0 mA for the 200 mM Tricine buffer. The sample was injected for 15 s. The sample volume was ~0.95 nl. Rinses were carried out under high pressure and sample injection carried out under low pressure. Each sample contained 0.03 pg of the fluorescent marker, 5-(biotinamidocapryolyamido)pentylthioureidylfluorescein (Sigma, St. Louis, MO, USA).

3. Results

Fluorescein iodoacetamide was chosen to label these peptides because this label reacts preferentially with free sulfhydryl groups at neutral pH values and, in this case, reacted with the free sulfhydryl on the cysteine without interference with the antigenicity of the peptide. The concentration of the fluorescein labeled pep1 (Fl-pep1) was 3.76 μM as determined by Lowry assay and 3.45 μM as determined by competition radioimmunoassay. The number of fluorescein molecules per molecule of pep1 was 0.82. For pep2 the concentration of the labeled peptide as determined by the Lowry method was 62.0 μM. The number of fluorescein molecules per molecule of pep2 could not be determined accurately because of unreacted dye remaining after P-4 column chromatography.

Immune complex formation was observed with purified IgG from the rabbit antiserum made to the two peptides, pep1 and pep2 from the prion protein (Fig. 1). Both peptides contained impurities that appeared later in the electropherogram (data not shown). Fluorescein labeled pep2 (Fl-pep2) contained a higher level of these impurities. Fl-pep1 migrated at 1.10 min (Fig. 1a). Immune complexes migrated at 1.02 min (Fig. 1b). Fl-pep2 migrated at 1.36 min (Fig. 1c) and immune complexes migrated

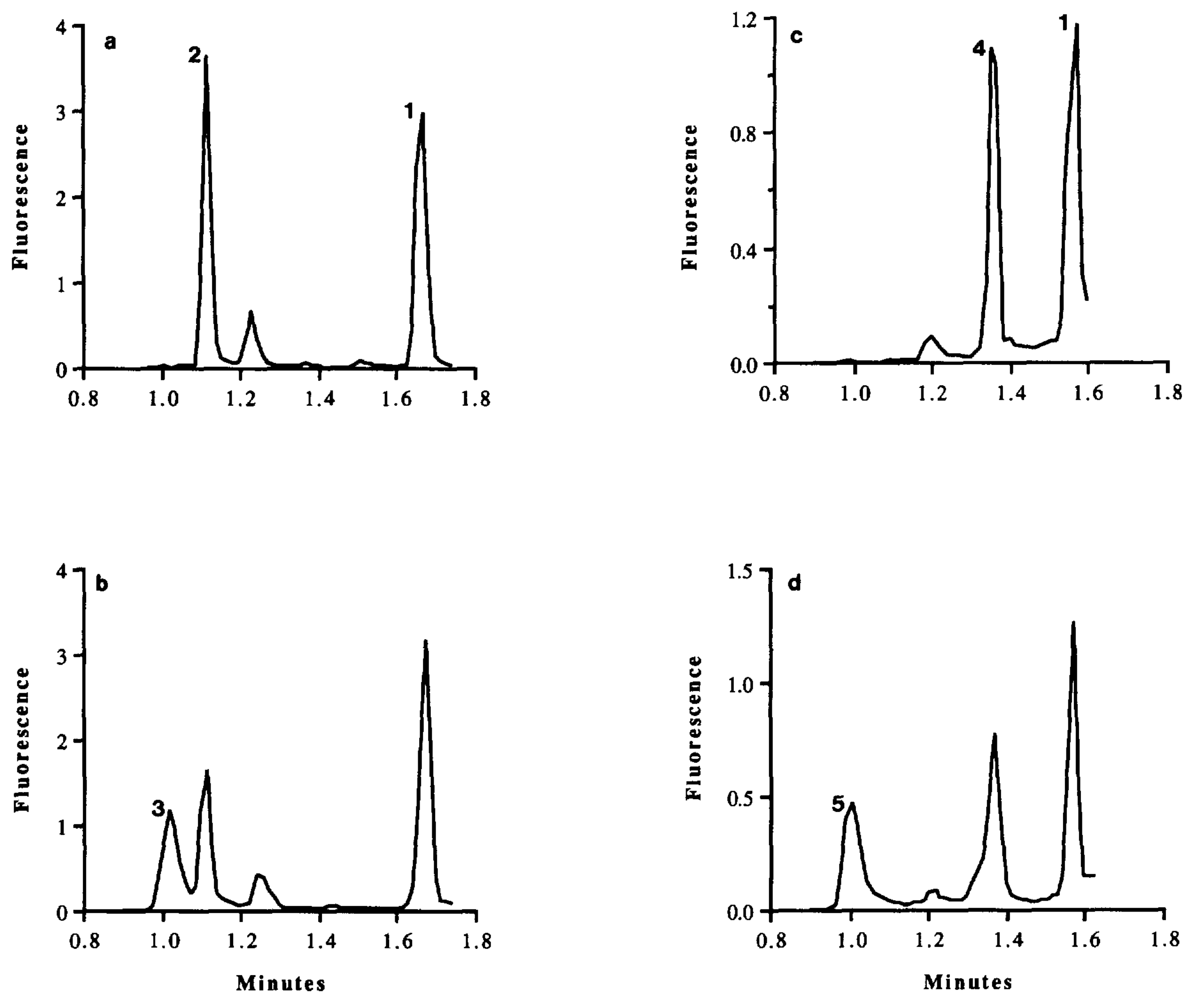

Fig. 1. Electropherograms showing the fluorescent labeled peptides and immunocomplexes in the presence of rabbit IgG made to each respective peptide. (a) Fl-pep1 (3.76 μM); (b) Fl-pep2+1.5 ng rabbit IgG; (c) Fl-pep2 (62.0 μM); (d) Fl-pep2+ng rabbit IgG. (1) Position at which the marker migrates; (2) position at which Fl-pep1 migrates; (3) position at which immune complex of Fl-pep1 migrates; (4) position at which Fl-pep2 migrates; (5) position at which immune complex of Fl-pep2 migrates.

at 1.01 min (Fig. 1d). The marker migrated at 1.56 min. The amount of immune complex formation was directly dependent on the amount of antibody present as shown in Fig. 2a and Fig. 2b. The competition of unlabeled peptide for binding with the fluorescein labeled peptides was concentration dependent (Fig. 3). Because pep1 was labeled with a higher efficiency than pep2, the concentration of unlabeled pep1 (Fig. 3a) required for competition was about 100-fold less than for pep2 (Fig. 3b). Fl-pep1 was used to assay the competition of the brain samples. The brain equivalents tested were equivalent (1:50 dilution), 0.66 (1:75 dilution), 0.5 (1:100 dilution) and 0.25 (1:200 dilution). Ratios for the bound/free at the concentration of antibody used in the assay ranged from 1.6 to 1.8. The ratios for brain samples from scrapie-infected sheep and lower dilutions of normal brain were below 1.0 (Fig. 4). The variation from sample to sample in the assays of brain material was similar to that in the other assays. This variation was 5–8%. An approximate amount of prion protein present in the brain material was calculated from the ratio of bound/free using a standard curve with known moles of unlabeled peptide. In this assay, the

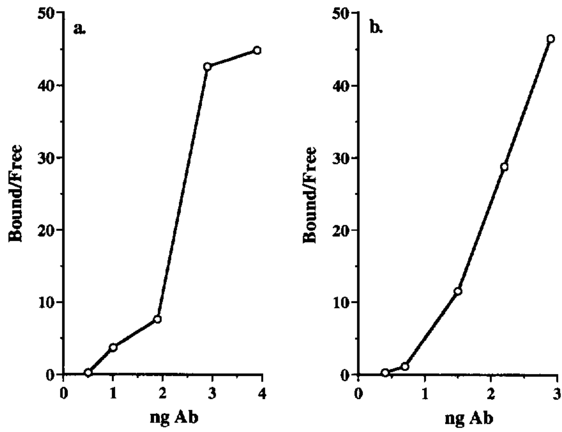

Fig. 2. Plot of the antibody concentration vs. the bound (represented by the immunocomplex peak)/free (represented by the labeled peptide peak). (a) Pep1; (b) Pep2.

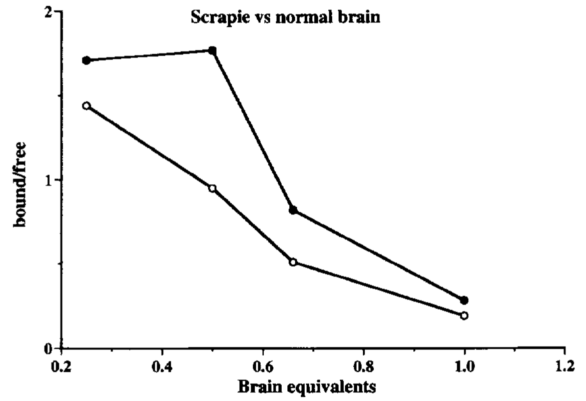

Fig. 4. Plot of the bound/free vs. the amount of scrapie-infected brain or normal brain equivalents. Fl-pep1 was used in this study. Bound/free ratios when with this concentration of antibody ranged from 1.6 to 1.8.

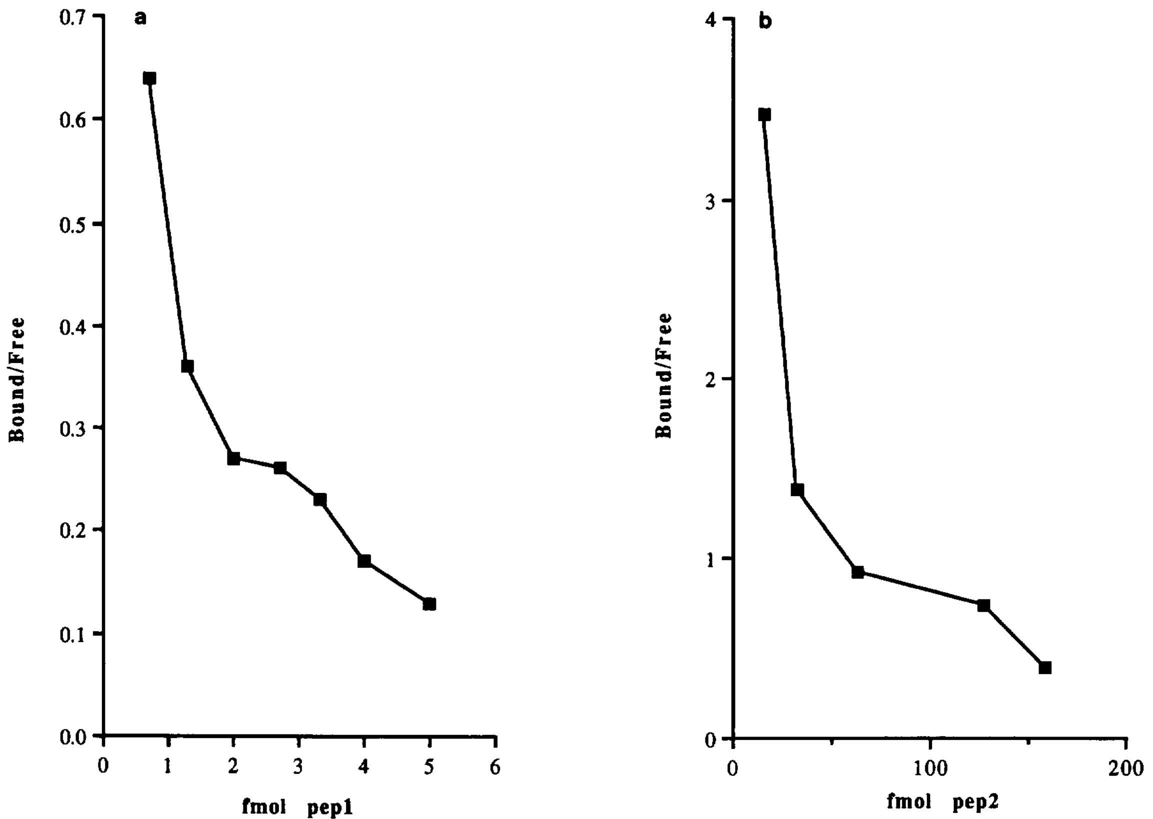

Fig. 3. Plot of the peptide concentration vs the bound/free. (a) Pep1; (b) Pep2.

amount of prion protein that was detected was calculated to be ~27 pg.

4. Discussion

The diagnosis of scrapie in sheep, as well as in other TSE, is made postmortem. This diagnosis is based on the histological identification of the lesions present in the brains of the animals. Recently, other diagnostic tests using immunohistochemistry [19] and immunoblotting techniques [23] have been used to show the presence of the prion protein, characteristic of this disease [24]. We have demonstrated [17,18] that CE is more sensitive and faster than the previous tests. Our approach of using labeled peptides from the prion protein increased the sensitivity of detection from μg range to the pg range. Further improvements in sensitivity can be made by incorporating fluorescein during peptide synthesis. This would limit the amount of unlabeled peptide that decreases the sensitivity of the assay and increase the efficiency of labeling. When the 200 m*M* Tricine buffer was used the peak shape was dramatically improved over those obtained with phosphate (data not shown) or borate buffer [18] systems. The area and peak heights were easily determined with confidence. The spikes and irregular peaks that were present in the borate buffer system were not present. Also purifying the antiserums over protein G decreased the incubation time for formation of the immune complexes from 16 h to 10 min [18]. Possibly, the sensitivity can be increased even further by producing Fab fragments of affinity purified antibodies or by using an affinity chromatography with each respective peptide. Another possibility is to enrich the prion protein in the brain samples by affinity chromatography prior to the electrophoretic separation and analysis. This technique worked well for the detection of insulin from single cells [25]. Because of the enhanced sensitivity, some of the residual normal protein from which the prion protein is derived was detected using Fl-pep1. When higher concentrations were used of the labeled peptide [18] more prion protein (competing antigen in the brain samples) was required to compete. Normal brain material did not show competition at this higher concentration of Fl-pep1. When Fl-pep2, which was approximately 100-fold more concentrated than Fl-pep1, was used to assay prion protein in the brain samples, at least 100-times more of scrapie-infected brain material was needed to show competition and the normal brain material did not compete. Other techniques for detection of the prion protein are not sensitive enough to detect the undigested normal protein in these samples. Other components in the brain samples, including other proteins, peptides and fatty acids, may also contribute to this problem [26]. Additional protease treatment of the samples may eliminate more of the normal protein. The prion protein does become protease sensitive after treatment with the SDS [10]. These improvements in sensitivity make CE a method that can now be used to study the pathogenesis of the disease caused by the prion proteins. This is not possible with other available methods because they are not able to detect small amounts of prion protein that are thought to be present early in the infection. The small amount of scrapie-infected material needed reduces risks involved in handling potentially infectious materials (prion protein is inactivated by SDS treatment) and makes possible the use of biopsy samples for the detection of the prion protein. As a result, this CE technique has the potential for wide application in determining the presence of prion protein in both animal and human samples.

Acknowledgments

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