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Capillary isoelectric focusing of the scrapie prion protein

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Abstract

Prion diseases or transmissible spongiform encephalopathies belong to a group of neurodegenerative diseases that infect both animals and humans. These diseases are associated with an accumulation of fibrils in the brains of infected individuals. These fibrils are composed of an abnormal isoform of a host-encoded glycoprotein that is characterized by its insolubility and partial resistance to proteases. Another characteristic of the scrapie prion protein (PrP^{sc}) is the wide range of isoelectric points (p*I* values) that have been observed on conventional isoelectrofocusing gels. In this study, we explored the use of capillary isoelectric focusing (cIEF) to characterize the p*I* values for PrP^{sc} isolated from sheep and hamster brain. We used a Beckman 5500 P/ACE using UV detection at 280 nm. A cIEF 3–10 Kit from Beckman Instruments was used to perform the analysis. The PrP^{sc} was solubilized in 0.01 *M* Tris–HCl, pH 8.00 containing 2 m*M* EDTA, 5% SDS and 10% hexafluoroisopropanol at 100°C for 10 min. The solubilized PrP^{sc} was placed over a high-performance hydrophilic interaction column. After elution, the peaks were concentrated and assayed for immunoreactivity with specific antisera. The peaks that contained immunoreactivity were then placed on the cIEF capillary. The samples containing PrP^{sc} were solubilized in 1% *n*-octylglucoside before isoelectric focusing. The scrapie infected sheep sample had peaks with p*I* values ranging from 5.2 to 3.00 with a major peak at 3.09. The normal sheep brain had p*I* values that were higher. The hamster adapted scrapie strain had peaks with p*I* values ranging from 6.47 to 3.8. These p*I* values were slightly higher than those obtained for the sheep samples. The use of cIEF to determine the p*I* values of PrP^{sc} led to the identification of a major species of PrP^{sc} from sheep with a very acidic p*I*. © 1998 Elsevier Science B.V.

Keywords: Isoelectric focusing; Proteins; Prion proteins

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are a family of diseases found in animals and in humans. These diseases cause disorders of the central nervous system and eventually result in death [1]. Scrapie, the prototype of this family, in sheep and goats has been known for over 200 years [2]. Recently, TSEs have become the focus of much interest because of the outbreak of bovine spongiform encephalopathy in the British Isles [3]. Most of the data suggest that the disease agent is an abnormal form of a normal host glycoprotein [4,5]. The abnormal protein (PrP^{sc}) is a conformational isomer of the normal protein [6]. When an immunoblot is made of samples prepared from infected animals three isoforms are noted [7]. These are ascribed to the differences in glycosylation of PrP^{sc} [7]. Reports in the literature have shown that preparations of PrP^{sc} have isoelectric points (p*I* values) ranging from 4.0 to 8.0 [8–12]. The starting material in these reports was either a mouse or a hamster adapted scrapie strain from sheep. The amount of PrP^{sc} produced in the brains of the hamsters or mice

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by the scrapie adapted strains is considerably more than PrP^{sc} produced in the brains of naturally infected sheep. The methods used to do conventional isoelectric focusing require much more material than would be generally found in sheep brain. Because of the inherent sensitivity of capillary isoelectric focusing (cIEF) [13,14], we investigated this technique to characterize the p*I* values for sheep PrP^{sc}. We found that by addition of 1% *n*-octylglucoside, sheep PrP^{sc} could be solubilized in the gel buffer provided. The p*I* values found for the sheep PrP^{sc} ranged from 5.2 to 3.0. The major peak had a p*I* of 3.09. The results from cIEF were confirmed by conventional isoelectrofocusing gel electrophoresis and by immmunoblot.

2. Experimental

2.1. Preparation of sheep brain material

Scrapie infected brains were obtained from sheep that were confirmed positive by histological examination and by immunohistochemistry [15]. Normal sheep brains were obtained from a scrapie free flock and confirmed negative by the above tests. Hamster brains (strain 263K) were obtained from Dr. D. McKenzie. All of the brain material was prepared by a modification of Bolton et al. [16]. Briefly, for sheep, 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, 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.) For hamster brain, the same procedure was used as above except that the whole brain was used. The homogenate was centrifuged at 10 000 *g* to remove particulates. 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 containing 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). The PrP^{sc} in the sample was solubilized in 0.01 *M* Tris–HCl, pH 8.00 containing 2 m*M* EDTA, 5% sodium lauryl sulfate (SDS) and 10% hexafluoroisopropanol at 100°C for 10 min and applied to PolyWAX LP (Poly LC, Columbia, MD, USA) high-performance liquid chromatography (HPLC) column (200×4.6 mm) in 100% acetonitrile containing 0.1% triflouroacetic acid (TFA) and 50 m*M* hexafluoroisopropanol (buffer A). The flow-rate was 0.5 ml/min. The conditions for eluting PrP^{sc} were 100% A for 8 min; a linear gradient to 100% water containing 0.1% TFA and 50 m*M* hexafluoroisopropanol (buffer B) in 15 min; 100% B for 10 min. Peak fractions were collected, dried in a vacuum centrifuge (Savant Instruments, Farmingdale, NY, USA), resuspended in 10 μl of water and assayed for PrP^{sc} by immunoblot. The samples positive for PrP^{sc} were used in the isoelectric focusing assays.

2.2. Isoelectrofocusing gels

Precast isoelectrofocusing gels with a p*I* range from 3–9 were purchased from Pharmacia Biotech (Uppsala, Sweden). The gels were run on the

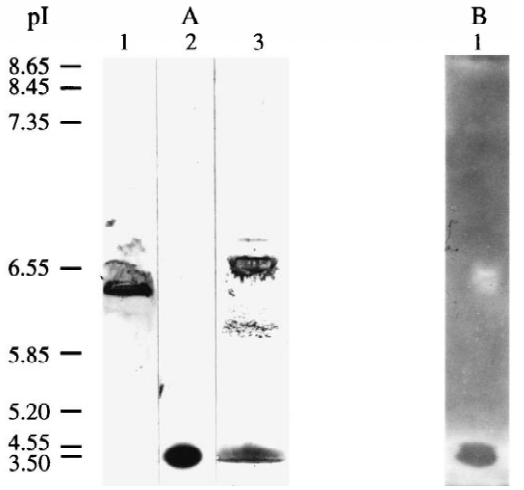

Fig. 1. Isoelectrofocusing gels. Four μl of each sample was used. Panel A: Silver stained and counter stained with Coomassie Brilliant Blue R. Lane 1=normal sheep brain; lane 2=scrapie infected sheep brain lane; 3=scrapie infected hamster brain. Panel B: Immunoblot of the scrapie infected sheep brain.

Phastsystem (Pharmacia Biotech) according the manufacturer's instructions. Isoelectrofocusing markers (Pharmacia Biotech) were run with each gel. The amount of sample that was applied to the gels was 4 μl. After running, the samples were stained with silver stain and then counterstained with Coomassie Brilliant Blue R (Research Products, Mount Prospect, IL, USA) in the Phastsystem. Immunoblot was done on the Phastsystem using diffusion for 20 min at 70°C. The nitrocellulose paper was pretreated with 25 m*M* Tris, 192 m*M* glycine containing 20% methanol and 50 m*M* hexafluoroisopropanol. After blotting, the nitrocellulose paper was incubated in 20 m*M* Tris pH 7.5 containing 500 m*M* NaCl, 0.05% Tween 20 (TTBS) and 5% fish gelatin (Sigma, St. Louis, MO, USA) for 1 h. The blot was washed 2× with TTBS and then incubated with a dilution of 1:750 of antibodies made to peptides of the prion protein for 3 h at 25°C. After incubation, the blot was washed 2× with TTBS and then incubated with biotinylated protein G (Bio-Rad Labs., Hercules, CA, USA) for 1 h. Again the blot was washed as above. Horse radish peroxidase coupled to NeutrAvidin (Pierce, Rockford, IL, USA) was added to the blot and incubated for 1 h at 25°C. After incubation the blot was washed 6× with TTBS. After washing, the blot was incubated in the SuperSignal®Substrate (Pierce) system for 10 min and then exposed to Kodak X-OMAT AR (Eastman Kodak, Rochester, NY, USA) X-ray film for 15 s.

2.3. Capillary electrophoresis

cIEF focusing was performed on a Beckman P/ACE 5500 (Beckman Instruments, Fullerton, CA, USA) controlled by P/ACE station software (Beckman Instruments). A cIEF 3–10 Kit (Beckman

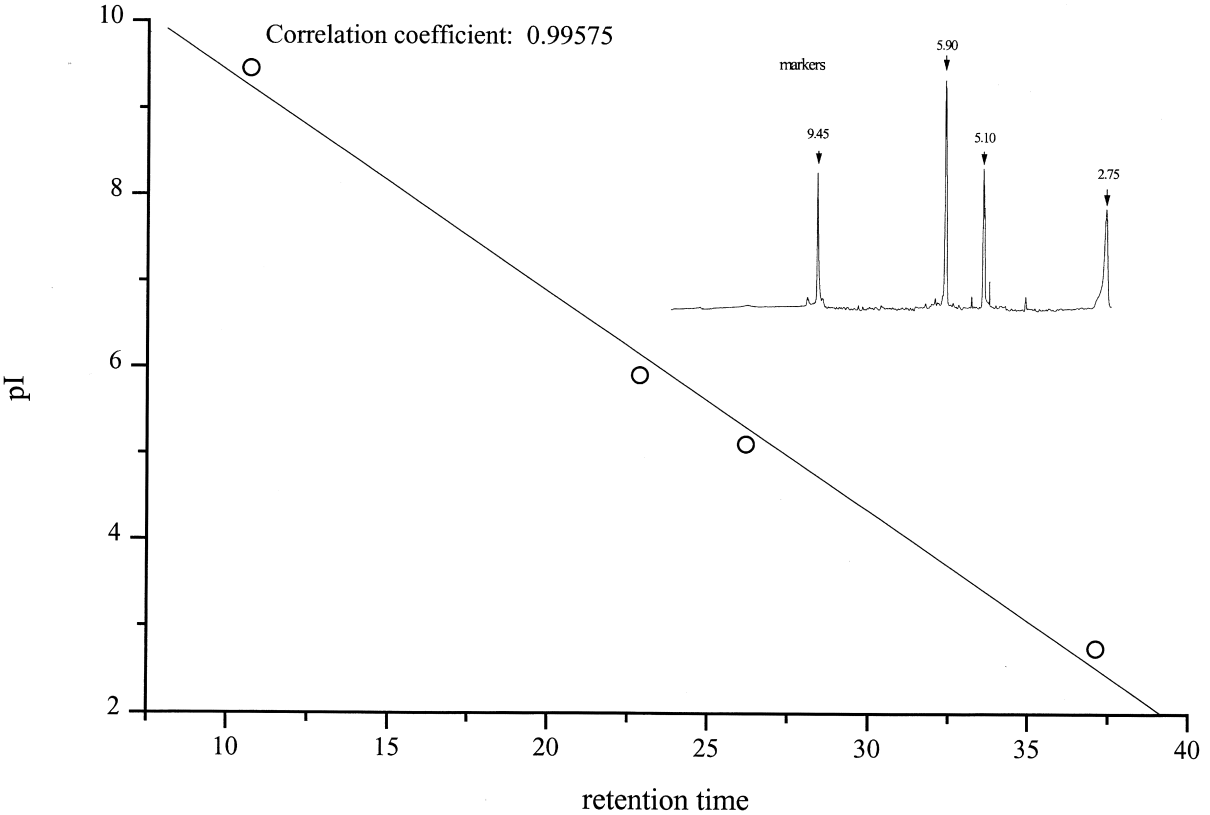

Fig. 2. Calibration curve made from the electropherogram of the protein standards. Inset: electropherogram of the standards. The standards included the proteins RNAase A, p*I* 9.45; carbonic anhydrase, p*I* 5.90; β-lactoglobulin, p*I* 5.10 and CCK peptide, p*I* 2.75.

Instruments) was purchased. The kit contained a neutral capillary 45 cm×50 μm, ampholytes ranging from 3–10, and cIEF gel. Markers, RNAase (p*I* of 9.45), carbonic anhydrase (p*I* of 5.98), β-lactoglobulin (p*I* of 5.10) and CCK flanking peptide (p*I* of 2.75) were included in the kit. The capillary was prepared by using a high-pressure rinse with 10 m*M* phosphoric acid for 2 min followed by a 10 min high-pressure rinse with filtered distilled water. To prepare the samples, 4 μl of the ampholyte solution was mixed with the cIEF gel. Five μl of the sample or markers was added to this mixture and mixed well and centrifuged at 7000 *g* to remove bubbles. The capillary was filled with the sample by a high-pressure injection for 1 min. The catholyte solution was 20 m*M* NaOH and the anolyte was 91 m*M* phosphoric acid in the cIEF gel. The proteins were then focused for 2 min at a voltage of 13.5 kV. To mobilize the focused proteins past the detector window, a low-pressure rinse was applied simultaneously while the field strength of the electric field was maintained at 500 V/cm. Using these conditions, the sample or markers were run for 34 min at 20°C with UV detection at 280 nm. To prevent the PrP^{sc} from precipitating in the cIEF gel, the samples were made 1% with *n*-octylglucoside (Boehringer Mannheim, Indianapolis, IN, USA). Five μl of purified brain samples from either the normal sheep or the infected sheep or hamster was used for the separation.

3. Results

The results of the isoelectrofocusing gels are shown in Fig. 1. The samples in panel A were stained with silver and counterstained with Coomassie Brilliant Blue R. The sample from the normal sheep brain had a band with a p*I* of 6.25 (panel A, lane 1). The sample from the scrapie infected sheep

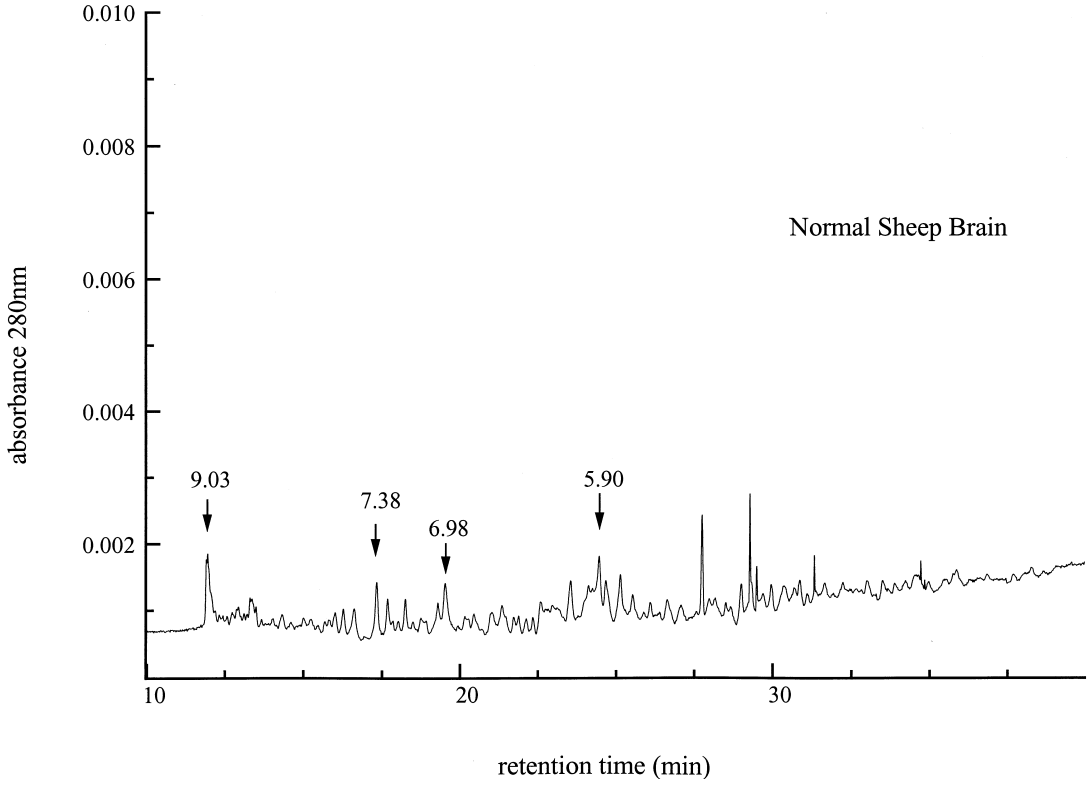

Fig. 3. Electropherogram of the normal sheep brain sample.

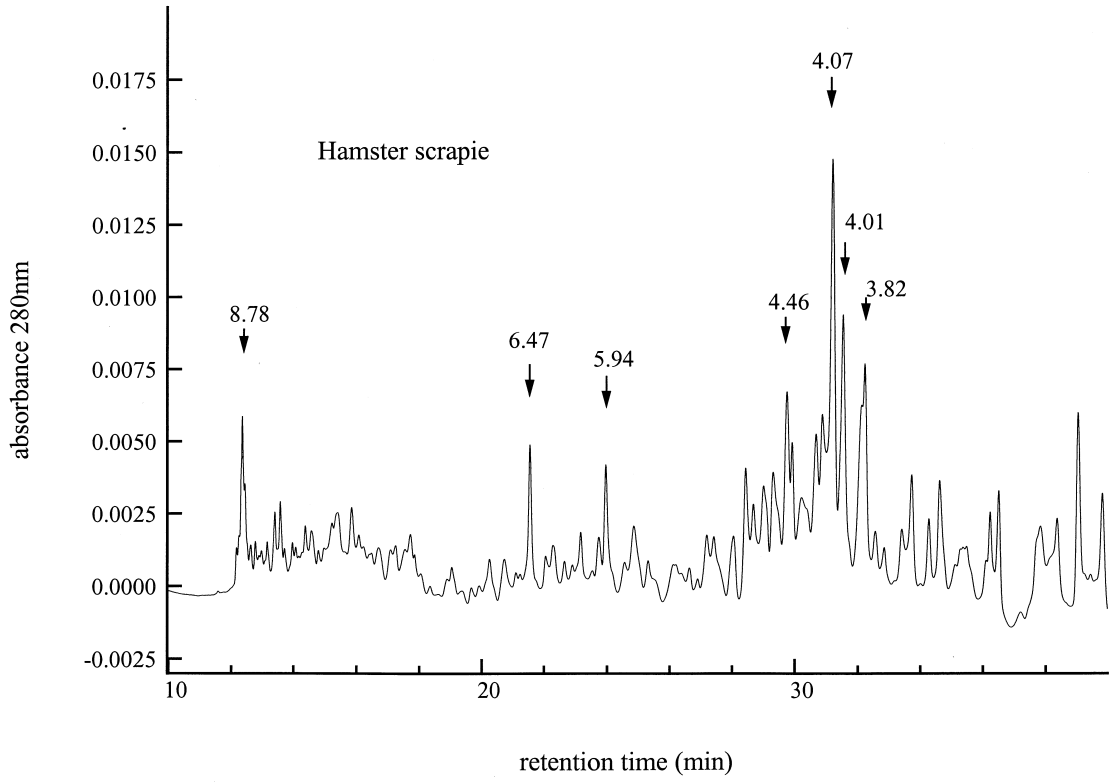

Fig. 4. Electropherogram of the scrapie infected hamster brain sample.

brain had a major band with a p*I* at ~3.0 (panel A, lane 2). When the sample was overloaded onto the gel, minor bands with p*I* values that ranged from ~4.0 to 6.0 were detected. Bands were also present in this range for the hamster adapted scrapie brain material (panel A, lane 3). A major band also was present at a p*I* of ~3.0. Immunoblot of the scrapie infected sheep brain is shown in Fig. 1 panel B, lane 1. The p*I* of the reactive bands is ~3.0. There appears to be two very closely migrating bands on the immunoblot which cannot be discerned on the stained gels of the isoelectrofocusing gel.

A plot of the calibration curve from the electropherogram of the markers is shown in Fig. 2. The actual electropherogram for the markers is shown in the inset of Fig. 2. The electropherogram of the sample from the normal sheep brain is shown in Fig. 3. The sample had very little protein present as can be seen by the low absorbance values. This made it difficult to assign p*I* values. No large peak was noted at a highly acidic p*I*. When hamster brain was used, the major peak had a p*I* of ~4.07 (Fig. 4). The other peaks were smaller in comparison. In the scrapie infected sheep brain, the major peak had a p*I* of ~3.06 (Fig. 5). Other minor peaks were noted from ~p*I* values of 3.5 to 5.5. These p*I* values agree with those estimated on gel isoelectrofocusing.

4. Discussion

Characterization of PrP^{sc}, the putative agent that causes TSEs is difficult because of the properties of this protein. However, elucidation of the pathogenesis is dependent upon this characterization. Although some progress has been made [17–19] in understanding the process, there are still many unknowns. Part of the confusion stems from the finding that the charge of this protein is so heterogeneous. Previous studies [12] of the distribution of scrapie infectivity using preparative agarose isoelectric focusing showed that most of the material did not migrate

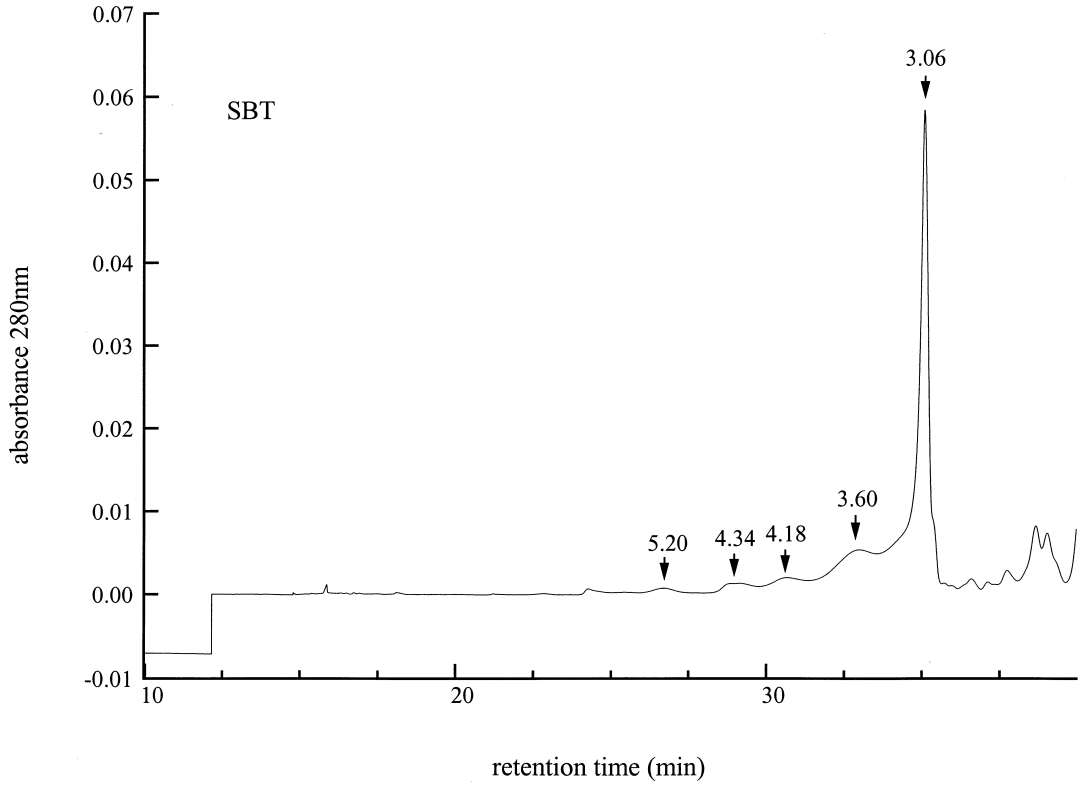

Fig. 5. Electropherogram of the scrapie infected sheep brain.

from the trough in which it was loaded. This material also was positive upon immunoblot. The infectivity was found throughout the alkaline regions of the gel. Other earlier studies [8,9] suggest that most of the prion protein was found at lower p*I* values. Another study [9] postulated that one of the major isoforms involved had a p*I* that was either too basic or acidic to be measured. In another study [10], PrP^{sc} had basic p*I* values before treatment with proteinase K and after protease treatment, PrP^{sc} showed acidic p*I* values. In our experiments with conventional isoelectrofocusing and with cIEF, we found that the major protein had a rather acidic p*I* of ~3.00 which would be outside the measuring range of the techniques used by the above researchers. This protein had reactivity to antibodies made to peptides of the prion protein by immunoblot and by a capillary electrophoresis assay [20] using competition by PrP^{sc} for fluorescent labeled peptides from the prion protein for binding to antibodies made to the corresponding peptides. This is the first report of a species of PrP^{sc} with a rather acidic p*I*. This new information will help in tailoring methods that will contribute to better purification methods and characterization of the pathogenesis of the scrapie prion protein.

5. Disclaimer

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