

03-080-1
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BioResource International, Inc.

December 10, 2003

Docket No. 03-080-1
Regulatory Analysis and Development
PPD, APHIS
Station 3C71
4700 River Road Unit 118
Riverdale, MD 20737-1238

Dear Sir/Madam:

On October 31, 2003, the USDA issued a proposal to open the border to Canadian beef, less than six months after the first case of Bovine Spongiform Encephalopathy (BSE) was confirmed in Alberta, Canada. The USDA has established a public comment period on this proposal until Jan 4, 2004. On behalf of my company, BioResource International (BRI), I would like to take this opportunity to offer my comments on this matter. As Secretary of Agriculture Ann Veneman has stated: "The United States has a long history of having safeguards in place to prevent the introduction of BSE. Protection of the U.S. food supply is our top priority. This proposed rule change reflects a thorough review of the scientific evidence, which shows the risk to public health to be extremely low." We are in full agreement with Secretary Veneman's comments, and my company has recently sponsored peer-reviewed scientific research on the "Enzymatic Degradation of Prion Protein in Brain Stem from Infected Cattle and Sheep" which we believe will further assist the USDA in preventing the potential incidence of BSE and other Transmissible Spongiform Encephalopathies (TSE) in the United States. See attached "MAJOR ARTICLE", *Journal of Infectious Diseases*, 188:1782-89 (1December 2003).

A recent Harvard University risk analysis determined that if BSE were to enter into the U.S., the risk of BSE spreading would be low due to controls put in place in the late 1990's and "eventually, the disease would be eliminated from the U.S." However, as was seen in Canada, even with containment policies and actions in place, just one positive BSE case in one infected animal would cost billions of dollars in lost international trade. Prevention, by virtue of safe decontamination, will further strengthen containment measures already in place in the U.S. and mitigate any potential future losses stemming from the occurrence of BSE. Thanks to the foresight of the USDA, there is a safe prevention measure for TSE on the horizon. With funding from the USDA IFAFS Critical and Emerging Issues Grant and from the National Cattlemen's Beef Association, BRI has developed an effective and scientifically proven method that has the ability to decontaminate prion contaminated surfaces and materials. See attached FACT SHEET.

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BRI is a North Carolina-based biotechnology company dedicated to harnessing the natural power of enzymes to address health and safety concerns in agriculture and industry. Today, BRI is prepared to help in the development of practical prevention and decontamination measures against prion diseases such as BSE.

We request a meeting with the appropriate Federal agencies to develop a safe and effective prion decontamination process. Prion diseases such as Scrapie and Chronic Wasting Disease are already in the U.S., and BRI is seeking partners for testing of decontamination measures effective against these diseases. Further testing of the enzymatic activity of Versazyme™ in degrading BSE prions under safe laboratory conditions is also needed in preparation for safeguarding animals, animal products, equipment, facilities, and transportation from BSE and to help prevent the introduction of TSE's into the United States.

Sincerely,

A handwritten signature in cursive script, appearing to read "G. Shih".

Giles Shih, Ph.D.
President
BioResource International

FACT SHEET

[BioResource International (BRI) holds patent rights for a USDA Funded and Scientifically Tested Enzymatic Process for PRION DEGRADATION]

- **Prions - infectious agents involved in Transmissible Spongiform Encephalopathies (TSE) - are notoriously resistant to destruction. See Abstract, attachment 1, “Enzymatic Degradation of Prion Protein in Brain Stem from Infected Cattle and Sheep,” The Journal of Infectious Diseases, December 1, 2003, page 1.**
- **Prions are a major health concern worldwide, and as such the USDA/FSIS has requested “...scientifically based information on...methods that could prevent [TSEs]” such as Bovine Spongiform Encephalopathy (BSE), Chronic Wasting Disease (CWD), and Sheep Scrapie. See attachment 2, FSIS, “FSIS Current Thinking...,” page 17.**
- **Contaminated materials such as feed, feed additives, medical and processing equipment, facilities, transportation equipment, and organic and inorganic animal products are thought to spread Prions. See published research on the USDA Web site (www.usda.gov).**
- **BRI has received financial support for and completed testing of a patented enzyme process (U.S. Patent No. 6,613,505) - from North Carolina State University, BRI and the USDA (USDA-IFAS Grant No. 2001-52100-11503) - that meets the requests of the USDA/FSIS. See attachments 1&3.**
- **“Decontamination based on enzymatic breakdown would offer an attractive possibility either as a single method or in combination with other inactivation treatments....” See attachment 1.**
- **The ultimate effectiveness of the BRI enzymatic process on “...prion inactivation has to be tested in mouse bioassays...” and is underway and supported by funding from the National Cattlemen’s Beef Association. See attachment 1.**

ATTACHMENT 1

“Enzymatic Degradation of Prion Protein in Brain Stem from Infected Cattle and Sheep.” *Journal of Infectious Diseases*, 188:1782-89.

December 1, 2003

Enzymatic Degradation of Prion Protein in Brain Stem from Infected Cattle and Sheep

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Prions—contagious agents involved in transmissible spongiform encephalopathies—normally survive proteolytic and mild protein-destructive processes. Using bacterial keratinase produced by *Bacillus licheniformis* strain PWD-1, we tested conditions to accomplish the full degradation of prion protein (PrP) in brain-stem tissue from animals with bovine spongiform encephalopathy and scrapie. The detection of PrP^{Sc}, the disease-associated isoform of PrP, in homogenates was done by Western blotting and various antibodies. The results indicated that only in the presence of detergents did heat pretreatment at >100°C allow the extensive enzymatic breakdown of PrP^{Sc} to a state where it is immunochemically undetectable. Proteinase K and 2 other subtilisin proteases, but not trypsin and pepsin, were also effective. This enzymatic process could lead to the development of a method for the decontamination of medical and laboratory equipment. The ultimate effectiveness of this method of prion inactivation has to be tested in mouse bioassays.

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders of humans and animals. In clinical cases, infectivity is accompanied by the conversion of the host-encoded prion protein PrP^C into a structurally altered form, PrP^{Sc}. No differences in primary structure have been found between PrP^C and PrP^{Sc} [1]. Although PrP^C occurs in both healthy and diseased tissues, the presence of PrP^{Sc} is typical for the diseased state and is biochemically detectable as PrP^{Sc}. PrP^{Sc} has a high content of β -sheet conformation, is partially resistant to proteinase K digestion, and is believed to exist as an aggregate [2, 3]. In the presence of detergents, PrP^{Sc} and infectivity can be effectively recovered from tissue homogenates at high centrifugal force, although the relationship between the 2 has not yet been clearly defined [4, 5].

The infective agent is highly resistant to treatments that destroy nucleic acids and is mainly, if not completely, composed of PrP^{Sc} [6]. In practice, contaminated materials are usually disinfected by boiling in 1% SDS under reducing conditions, boiling in 1 mol/L sodium hydroxide, or treatment with formic acid or bleach containing 20,000 ppm active chlorine [7–9]. These are harsh conditions. More compatible alternatives would be preferred, if possible. Strains of the agent differ in their resistance to heat inactivation, and the bovine spongiform encephalopathy (BSE) agent is one of the more resistant examples [9–13]. In addition to the conventional techniques mentioned, combined treatments of denaturation, heat, and/or high pressure are under investigation [16, 17]. Therefore, additional ways to inactivate BSE and other TSE agents would be highly desirable. They would be especially useful for the treatment of valuable medical instruments, laboratory equipment, and exchangeable items like contact lenses and dentistry tools [14, 15].

Decontamination based on enzymatic breakdown would offer an attractive possibility either as a single method or in combination with other inactivation treatments. Treatments that disrupt hydrogen bonds and favor solubilization would allow better degradation by proteases. Furthermore, the type of enzyme and di-

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gestion conditions are of crucial importance to obtain a high degree of inactivation. It has been found elsewhere that the proteolytic treatment of crude or partially purified scrapie from infected mouse and hamster brain poorly neutralizes infectivity [18–20], whereas, in purified hamster scrapie, proteinase K is capable of reducing infectivity by >4 orders of magnitude, although a considerable level of residual infectivity remained [20, 21]. For purified hamster scrapie material, pretreatment with heat, denaturants, or high pH made PrP^{Sc} [22] and infectivity highly susceptible to degradation by proteinase K [23]. By contrast, in crude homogenates of mouse brain, heat inactivation did not always lead to the complete protease sensitivity of PrP^{Sc}, although infectivity was destroyed [13]. It is possible that PrP^{Sc} in tissue may be more protected than in purified prion preparations from heat denaturation and protease degradation or that the protease used was not effective. Because solubilization, heat, and enzymatic digestion are physically different processes, the combined effect of each could result in enhanced inactivation.

Conditions were investigated to obtain a high-level degradation of PrP^{Sc} by a combined treatment of solubilization, heat denaturation, and enzymatic digestion. This was performed with tissue homogenates of BSE- and scrapie-infected brain stem. Because of its broad substrate proteolytic activity, the enzyme used for digestion was a feather-degrading keratinase, which was recently isolated from *Bacillus licheniformis* PWD-1 (PWD-1 keratinase) and has been well characterized and produced on a large scale [24, 25].

MATERIALS AND METHODS

Tissues. All activities were carried out in a pathogen class-3 facility, in agreement with European Union directives and the guidelines set out by the Spongiform Encephalopathy Advisory Committee of the United Kingdom. Brain-stem material was derived from adult cattle and sheep with or without clinical signs of disease. A diagnosis of BSE and scrapie was made on samples from the obex region by 3 methods for different characteristics: histopathology for vacuoles [26], immunohistochemical techniques for the deposition of PrP [27], and on homogenates for detection of PrP^{Sc} by Western blotting with the Prionics Check method [28].

Enzymes and determination of protease activity. PWD-1 keratinase was produced and purified as described elsewhere [24, 25]. Proteinase K from *Tritirachium album* was purchased as purified crystalline product from Merck. The following enzymes were all purchased from Sigma Chemical as purified and crystalline material: *Clostridium histolyticum* collagenase type VII, hog pancreas elastase type III, hog stomach pepsin, bovine pancreas trypsin, *B. licheniformis* alcalase, and *Bacillus subtilis* subtilisin Carlsburg. The protein substrates bovine tendon collagen (Sigma type V), bovine neck ligament elastin, and bovine

casein were all purchased as powder from Sigma Chemical. Feather keratin was prepared by grinding and ball milling, as described elsewhere [24].

All enzyme activities were measured at their individual optimum conditions (according to Sigma Chemical manuals). The hydrolysis rate of keratin, elastin, and collagen was measured by a ninhydrin color reaction (absorbance at 590 nm) of increased free amino groups [24]. The casein hydrolysis rate was measured by the increased absorbance at 280 nm in the supernatant [29]. Relative activities of individual proteases were compared and calculated for each substrate, using as unity the average value obtained with the related specific enzyme (i.e., keratinase on keratin, elastase on elastin, etc.).

Tissue treatment. Pieces of 0.3–0.6 g of brain-stem material, cut from within 5 cm of the obex region were homogenized with an Omni International TH mixer with a disposable probe in 10× vol of 2% (wt/vol) *N*-lauroylsarcosine (Sigma) in 0.1 mol/L sodium phosphate (pH 7.2; lysis buffer). When specified, *N*-lauroylsarcosine was replaced by a mixture of 2 detergents, 0.5% sodium deoxycholate and 0.5% Triton X-100 in 50 mmol/L Tris-HCl. In some experiments, tissue was first cut into small blocks, divided in 2 aliquots, and either homogenized in lysis buffer or first heated before homogenization in lysis buffer. The heating of homogenates was performed for 40 min at 115°C (pressure, 72 kPa) in a Vulcain pressure cooker, unless specified otherwise. The escape of aerosols from the vials was prevented by sterilization stoppers made of filter paper. In experiments to evaluate the effect of different temperatures <115°C, homogenates were heated for 40 min in 2-mL screw-cap vials in a temperature-controlled heating block with tightly fitting holes. Homogenates were stored at 4°C–8°C unless otherwise specified.

Enzymatic digestion of brain-stem homogenates. For PWD-1 keratinase, digestions were typically done at 50°C for 1 h with 100 µL of tissue homogenate and 10 µL of purified enzyme at a final concentration of 200 µg/mL in a 96-well polypropylene digestion plate designed for polymerase chain reaction. Reactions were stopped by the addition of 10 µL of 12.5 mmol/L Pefabloc SC (Roche). Digestions with other proteases were done under the same conditions, except that temperature of incubation was different depending on the enzyme used: proteinase K, 50°C; alcalase and subtilisin Carlsburg, 35°C; and trypsin and pepsin, 37°C. In the case of pepsin, the homogenate was first adjusted to pH 2–3 by the addition of 4 µL of 0.1 mol/L HCl, and, at the end of the digestion, the pH was readjusted to pH 7–8 by the addition of 8 µL of 1 mol/L Tris.

Gel electrophoresis, Western blotting, and immunodetection of PrP. If not specified, all samples were precipitated by the addition of 9× volume of methanol and subsequent centrifugation. Pellets were subsequently taken up by thorough trituration in 100 µL of sample buffer, as described in a manual

for NuPage gels from Invitrogen. Electrophoresis in SDS-PAGE, the Western blotting procedure, PonceauS staining for total protein, and immunostaining with PrP-specific antibodies were done according to established procedures [28, 30–32]. Prion protein was detected with primary antibody 6H4 [33] at 0.2 $\mu\text{g}/\text{mL}$ and rabbit anti-mouse IgG antibody conjugated to alkaline phosphatase (DAKO). In some cases, additional PrP detection was done with other PrP-specific antibodies at concentrations that would warrant the detection of traces and various epitopes of bovine PrP antigen: rabbit polyclonal antibody R505 (raised with ovine PrP peptide 100–111) at 1:2000 dilution, and 4 different murine monoclonal antibodies each at 1 $\mu\text{g}/\text{mL}$: 6H4, 66.94b4 (raised at Central Institute for Animal Disease Control, Lelystad, The Netherlands, with human and bovine recombinant PrP, which binds to a conformational epitope of the proteinase K-resistant part of PrP^{Sc}), F99/97.6.1 (raised with ovine PrP peptide 217–231), and 12F10 (raised with DNA coding for human PrP; it binds to synthetic peptide 141–159 from the human PrP sequence; SPI-BIO) [34–37]. The development of the signal was done with CDP-Star, according to the supplier's instructions (Tropix), and signals were recorded on photographic film (Eastman Kodak). In addition to molecular-weight markers, 2 controls were used. First, a BSE-infected brain stem was homogenized and, without heat pretreatment, digested with proteinase K, according to the Prionics Check protocol (sample P). This showed the migration of PrP^{res}, the typical protease-resistant fraction of PrP^{Sc}, which consists of a diglycosylated, monoglycosylated, and unglycosylated protein band. Second, a nondigested brain-stem homogenate from a healthy cow (sample N) served as a control, to show the migration of intact PrP^C and PrP^{Sc}. There was no difference seen in the migrational behavior between these 2 isoforms when they were left intact. Molecular-weight markers, mixed with the control, were carbonic anhydrase from bovine erythrocytes (29 kDa; Sigma) and β -lactoglobulin from bovine milk (18.4 kDa; Sigma). In some experiments, the density of complete (top to bottom) lanes was recorded from the film with an Agfa Duo T200XL scanner and further processed with GelPro software.

RESULTS

General comparison of different proteases with PWD-1 keratinase. Purified PWD-1 keratinase from *B. licheniformis* PWD-1 was tested for its general capacity to break down proteins known to be poorly hydrolyzed by proteases. PWD-1 keratinase exhibited excellent proteolytic properties in digesting keratin, elastin, collagen, and casein, compared with other proteases like elastase, collagenase, proteinase K, and trypsin (data not shown). PWD-1 keratinase was therefore further used for the prion-degradation studies.

Degradation of PrP^{Sc} by keratinase in cattle and sheep tis-

sue. It appeared that, when tested on BSE-infected brain-stem material homogenates that were not heat treated, PWD-1 keratinase, like proteinase K, was not capable of degrading the PrP^{Sc} beyond the typical PrP^{res} product, with a banding pattern of diglycosylated, monoglycosylated, and unglycosylated PrP forms (figure 1A, sample P and lanes 13–16). Prion protein in negative material was fully digested. However, when the BSE-positive homogenates were preheated at 115°C in a pressure cooker, PWD-1 keratinase fully abolished the immunodetectability of PrP^{Sc} (figure 1A, lanes 9–12). It appeared that the distribution of PrP^{Sc} can vary between the soluble and insoluble fractions, especially after the freezing of brain homogenates; this possibly could influence the resistance to proteolytic degradation. Therefore, we tested whether all PrP was digested from both whole homogenate and the soluble fraction only, both in unfrozen and frozen aliquots. Indeed, PrP^{Sc} degradation by keratinase was effective for both whole homogenates, the supernatant, and frozen aliquots thereof (e.g., figure 1A, lanes 9–12). This high extent of proteolytic degradation was not only established with antibody 6H4 at a high concentration (1 $\mu\text{g}/\text{mL}$) but also with 4 additional antibodies, R505, 66.94b4, 12F10, and F99/97.6.1, which have different specificities for PrP^{res} (data not shown). Few coagulates were seen in the vials after heating in the presence of detergent, and, possibly as a result, only in nondigested samples was immunoreactive material observed at the top of gel lanes. A PrP-positive protein with properties of PrP^C was observed when the enzyme was omitted from the digestion mixture, which indicates that most of the PrP protein was dissociated in its monomeric form in the samples applied to the gel (figure 1B, lanes 3–4; figure 1C, lanes 1–2). These experiments were performed in the presence of 2% (wt/vol) of the detergent *n*-lauroylsarcosine. To obtain full digestion, the presence of the detergent was essential. Protease-resistant PrP^{Sc} remained detectable in BSE-positive homogenates when heating was carried out in the absence of detergent, whereas normal PrP was fully digestible in BSE-negative tissue (figure 1B, lanes 1–2 lanes 5–6). Different types of detergent used in the homogenate during the heating step had different effects on the extent of subsequent digestion. When 2% *n*-lauroylsarcosine was used, PrP^{Sc} was fully digested; when a mixture of Triton X-100 and sodium deoxycholate was used, residual PrP^{res} was observed (figure 1C, lanes 3–4). Effective digestion after preheating in the presence of *n*-lauroylsarcosine occurred in all samples tested: BSE- and scrapie-infected brain stems from cattle and sheep, respectively (figure 2).

Optimization of PrP^{Sc} digestion with keratinase. PWD-1 keratinase at a concentration >10 $\mu\text{g}/\text{mL}$, digested PrP for 60 min to levels below detection, whereas, in comparison, proteinase K was somewhat more active, yielding such results at enzyme concentrations >5 $\mu\text{g}/\text{mL}$, compared with proteinase K (figure 3A). Under suboptimal enzyme concentrations like 10 $\mu\text{g}/\text{mL}$,

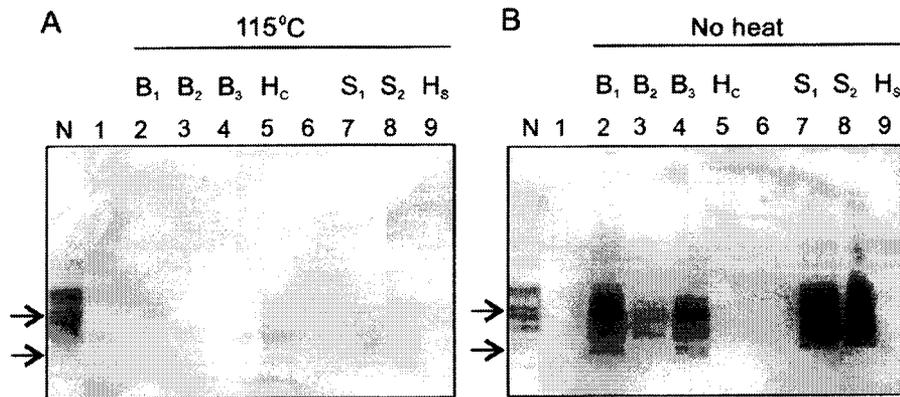


Figure 2. Digestion with keratinase of heated and unheated bovine spongiform encephalopathy (BSE) and scrapie samples from different individual animals is shown. Heat pretreatment is indicated. None of the samples was precipitated with methanol prior to loading. B₁, B₂, and B₃, samples from 3 different BSE-infected cattle; H_c and H_s, samples from healthy cow and sheep, respectively; N, sample containing nonheated nondigested brain-stem material from a healthy cow (control for intact PrP); S₁ and S₂, samples from 2 different scrapie-infected sheep. Lanes 1 and 6 are empty. Tissue equivalents applied: 400 μ g, except lane N, 200 μ g. Arrows indicate 29- and 18.4-kDa molecular-weight markers.

temperature and pH, proteinase K, alcalase, and subtilisin Carlsburg were able to fully degrade PrP from heated brain-stem homogenates, whereas, without heating, the typical PrP^{res} pattern appeared for each of these enzymes (figure 5). PrP^{res} digestion with trypsin (and pepsin at pH 2.4 and 37°C; data not shown) were incomplete on heated samples; hardly any degradation occurred on unheated material.

DISCUSSION

The present study demonstrates that it is possible to destroy PrP^{Sc} in infected crude brain material toward immunochemically undetectable levels. The theoretical basis for the result is that the aggregated and β -sheeted structure of the rogue PrP protein was first denatured by heat in the presence of detergent and subsequently digested with a broad-spectrum serine endopeptidase. The case is similar to the keratinase degradation of feather keratin, which is also rich in β -sheet structure. In addition to PWD-1 keratinase, proteinase K and 2 proteases of the subtilisin family were found to be capable of degrading PrP. It is assumed that this method can reduce infectivity at least 3 orders of magnitude and has the potential to accomplish complete inactivation.

The effectiveness of the method was monitored by Western-blot assay, a test that is dependent on the detection by PrP-specific antibodies and recovery of the BSE agent (i.e., the solubility of the material). Recently, immunochemical tests for the detection of PrP^{Sc}, like Western blotting, have been found to be suitable for the routine diagnosis of BSE in the brain-stem tissue of cattle [28, 38]. The sensitivity of such immunochemical tests appears to be comparable with or better than bioassays with unpassaged BSE material [39]. The limitation of this comparison, however, should be kept in mind. BSE testing in conventional

mice is relatively insensitive, reaching variable titers in the range of $10^{2.7}$ – $10^{5.2}$ ID₅₀/g of brain tissue [40, 41]. More-sensitive test systems for unpassaged BSE have been developed in transgenic mice that express multiple copies of the bovine PrP gene. These systems reach titers of 10^7 – 10^8 ID₅₀/g of brain-stem tissue [42, 43]. For sheep scrapie, however, such a reproducible and high-titer mouse bioassay is still not available.

The combination of denaturation by heat and subsequent enzymatic treatment to destroy PrP^{Sc} is potentially an adaptable procedure to eliminate prions from contaminated equipment. Harsh chemicals are not involved in this method. The presence of a detergent like *N*-lauroylsarcosine is required for the full digestion of PrP^{Sc}, presumably because the detergent facilitates both its solubilization and denaturation during the heating pretreatment. For surface cleaning, these are normal conditions in hospital and laboratory equipment. For the production of food proteins from animal byproducts, however, low-cost natural substitutes for the detergent should be sought. Heating >80°C is indispensable for the elimination of any microbial organisms. To obtain full proteolytic susceptibility, only preheating at 115°C was effective in the presence of *N*-lauroylsarcosine, and this temperature provided less-satisfactory results with the weaker detergents Triton X-100 and sodium deoxycholate, in which some PrP^{res} remained after subsequent digestion with keratinase. Potential improvements are to be expected in the future by a balanced regimen of denaturing temperature, choice of detergent, and enzymatic digestion. However, a safe temperature for pretreatment (115°C) to obtain an optimal enzymatic degradation of PrP^{res} is, in fact, a temperature that even is too high for potential proteases from thermophilic microorganisms.

The infectivity and protease resistance of PrP^{Sc} present themselves as 2 different properties of the TSE agent, and the re-

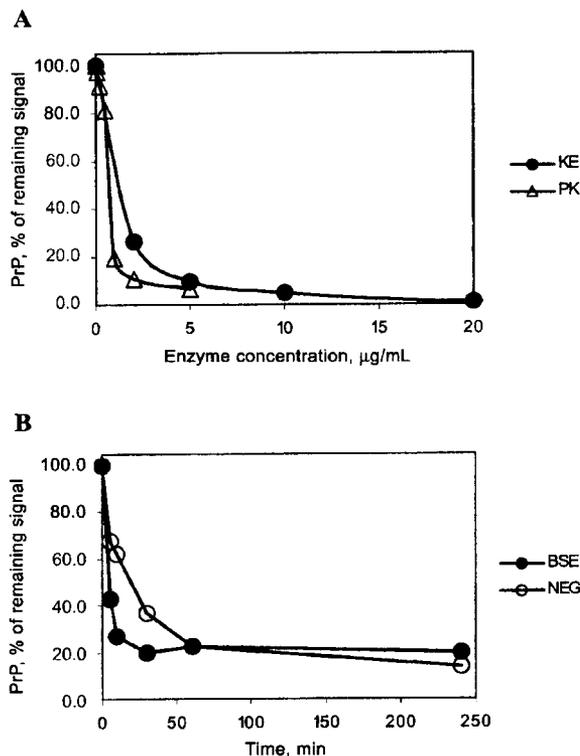


Figure 3. The effects of concentration and incubation time on keratinase (KE) digestion of prion protein (PrP) in heated brain-stem homogenate are shown. *A*, Bovine spongiform encephalopathy (BSE)-infected brain-stem homogenates digested at variable KE and proteinase K (PK) concentrations during a fixed incubation time of 60 min. *B*, The extent of digestion of PrP in BSE-positive (BSE) and -negative (NEG) tissue after varying time of incubation. Aliquots of 10% brain-stem homogenate were digested by KE PWD-1 at suboptimal concentration of 10 µg/mL. For both panels, photographic films of Western blots were recorded by a scanner, and the resulting densities further processed with GelPro software. Each data point is an integrated recording of a complete lane and is expressed as the percentage of the density signal obtained for 0 time incubation samples.

relationship between PrP^{Sc} and in vivo infectivity has not been clearly defined. Somerville et al. [13] reported that, in mouse brain homogenates infected with either of 2 adapted scrapie strains, ME7 and 22C, proteinase K-resistant PrP^{Sc} survived heating in the absence of detergent, although infectivity was reduced by >99.9%. The favorable use of detergent during heating to improve the proteolysis of PrP^{Sc} was shown: protease-resistant PrP^{Sc} remained when heating was done in the absence of detergent (figure 1*B*), but PrP^{Sc} was fully susceptible to keratinase when detergent was present. We postulate that, when homogenates are treated according to the presented procedure using effective detergents for extraction combined with proteolysis, infectivity like that of PrP^{Sc} will be more effectively destroyed than when it is heated in the absence of detergent.

It could be that the agent, if it is completely composed of PrP^{Sc}, would exist in at least 2 different multimeric β -sheeted forms—one an unaggregated structure and the other as an aggregated fraction—like amyloid with Congo red affinity [44]. Although both forms are infective and the infectivity is most susceptible to heat, their proteolytic susceptibility is dependent on the deaggregated and denatured state caused by heat in the presence of detergent. Proper denaturation during heating might be obtained only in the presence of an effective detergent (e.g., SDS or sarcosyl) or possibly other chaotropic conditions common in protein research (e.g., low pH or the use of urea or guanidium).

PrP^{Sc} in (nondenatured) brain homogenates from infected animals can be partially digested by proteinase K such that the characteristic conversion occurs of PrP^C into PrP^{res} [45, 46]. This molecular-weight transition has been a long-standing benchmark for the presence of PrP^{Sc}. Our data show that other proteases, such as subtilisins and PWD-1 keratinase but not trypsin and pepsin, also have this capacity. In addition, keratinase, proteinase K, and the 2 other subtilisins, although they are enzymes from different sources and with different amino-acid sequences, also yield the same-sized PrP^{res} bands. This phenomenon of a similar end result of proteolysis might be based on the conformational properties of PrP^{Sc} that determine the extent of its digestion for these serine endopeptidases.

The present study is a biochemical test that shows an effective erasure of prion protein. It promises new avenues for a decontamination or inactivation process, which is highly needed [47, 48]. Of basic significance, however, is the in vivo bioassay. BSE from cattle or as propagated from mice has been found to be resistant to heat inactivation [10, 11]. Experiments are in prep-

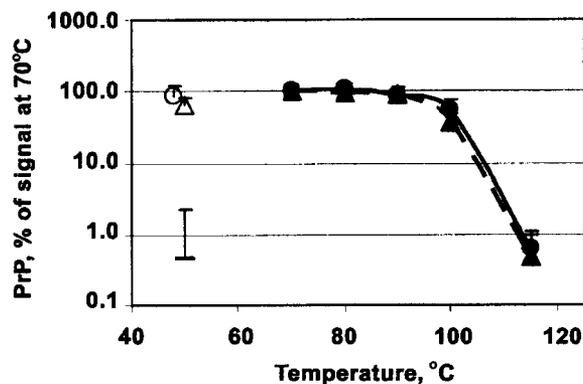


Figure 4. Different temperatures during the heat pretreatment of tissue homogenates from a bovine spongiform encephalopathy (BSE)-infected cow and a scrapie-infected sheep are shown. Data points represent average values from 3 digestion experiments plus SD and are expressed on the logarithmic axis as the percentage of the prion protein (PrP) signal obtained with the sample preheated at 70°C. *White symbols*, not preheated; *black symbols*, preheated at the temperature indicated; *circles*, BSE infected; *triangles*, scrapie infected; *bar*, a sample from healthy cow.

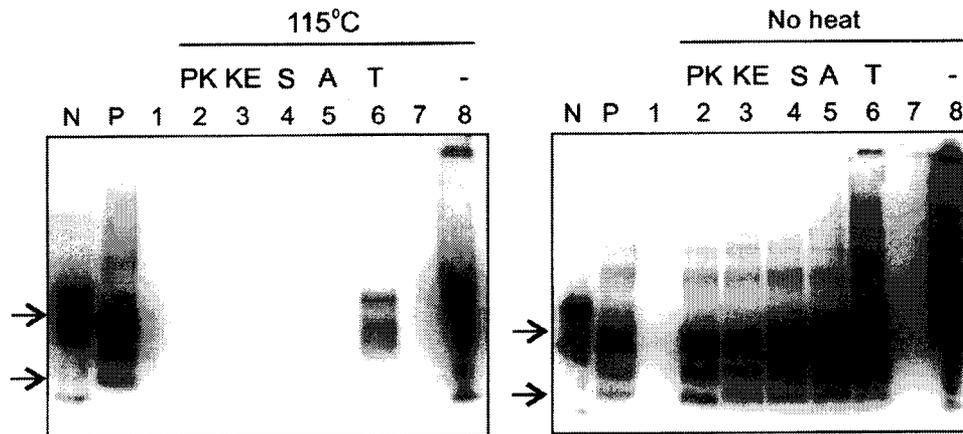


Figure 5. Digestion of prion protein (PrP)^{Sc} in bovine spongiform encephalopathy (BSE)-infected brain-stem homogenates by various enzymes: A, alcalase; KE, PWD-1 keratinase; PK, proteinase K; S, subtilisin Carlsburg; and T, trypsin. Heat pretreatment is indicated. The tissue equivalents applied were 1000 µg/lane, except for lane N, 200 µg. Lanes P and N are controls, as described in figure 1 and Materials and Methods. Lanes 8, no enzyme; lanes 1 and 7, empty.

aration to verify the biological effectiveness of this method with BSE field isolates and with the high-titer BSE strain 301V in mice. These experiments require 2–3 years to obtain the answer. It is, however, of general importance that transgenic mice and in vitro cellular assays will become generally available for the testing of unpassaged TSE agents with respect to BSE, scrapie, chronic wasting disease, and Creutzfeldt-Jakob disease. Such assays ideally need to be sensitive, flexible toward the testing material, and of relatively short duration.

Acknowledgments

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References

1. Stahl N, Baldwin MA, Teplow DB, et al. Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. *Biochemistry* **1993**;32:1991–2002.
2. Caughey BW, Dong A, Bhat KS, Ernst D, Hayes SE, Caughey WS. Secondary structure analysis of the scrapie-associated protein PrP 27–30 in water by infrared spectroscopy. *Biochemistry* **1991**;30:7672–80.
3. Pan KM, Baldwin M, Nguyen J, et al. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci USA* **1993**;90:10962–6.
4. Meyer RK, McKinley MP, Bowman KA, Braunfeld MB, Barry RA, Prusiner SB. Separation and properties of cellular and scrapie prion proteins. *Proc Natl Acad Sci USA* **1986**;83:2310–4.
5. Hilmert H, Diringer H. A rapid and efficient method to enrich SAF-protein from scrapie brains of hamsters. *Biosci Rep* **1984**;4:165–70.
6. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. *Science* **1982**;216:136–44.
7. Bolton DC, McKinley MP, Prusiner SB. Properties and characteristics of scrapie PrP27–30 protein. In: Prusiner SB, McKinley MP, eds. *Prions: novel infectious pathogens causing scrapie and Creutzfeldt-Jakob disease*. London, Academic Press, **1987**:173–96.
8. Ernst DR, Race RE. Comparative analysis of scrapie agent inactivation methods. *J Virol Methods* **1993**;41:193–201.
9. Taylor DM. Inactivation of transmissible degenerative encephalopathy agents: a review. *Vet J* **2000**;159:10–7.
10. Schreuder BEC, Geertsma RE, VanKeulen LJM, et al. Studies on the efficacy of hyperbaric rendering procedures in inactivating bovine spongiform encephalopathy (BSE) and scrapie agents. *Vet Rec* **1998**;142:474–80.
11. Steele PJ, Taylor DM, Fernie K. Survival of BSE and scrapie agents at 200°C. In: *Program and abstracts of a meeting of the Association of Veterinary Teachers and Research Workers (Scarborough, United Kingdom)*. **1999**:21.
12. Dickinson AG, Taylor DM. Resistance of scrapie agent to decontamination. *N Engl J Med* **1978**;299:1413–4.
13. Somerville RA, Oberthür RC, Havekotte U, MacDonald F, Taylor DM, Dickinson AG. Characterisation of thermodynamic diversity between transmissible spongiform encephalopathy agent strains and its theoretical implications. *J Biol Chem* **2002**;277:11084–9.
14. Rohwer RG. Analysis of risk to biomedical products developed from animal sources (with special emphasis on the spongiform encephalopathy agents, scrapie and BSE). *Dev Biol Stand* **1996**;88:247–56.
15. Rutala WA, Weber DJ. Creutzfeldt-Jakob disease: recommendations for disinfection and sterilization. *Clin Infect Dis* **2001**;32:1348–56.
16. Brown P, Meyer R, Cardone F, Pocchiari M. Ultra-high-pressure inactivation of prion infectivity in processed meat: a practical method to prevent human infection. *Proc Natl Acad Sci USA* **2003**;100:6093–7.
17. Jung G, Jones G, Masison DC. Amino acid residue 184 of yeast Hsp104 chaperone is critical for prion-curing by guanidine, prion propagation, and thermotolerance. *Proc Natl Acad Sci USA* **2002**;99:9936–41.
18. Hunter GD, Gibbons RA, Kimberlin RH, Millson GC. Further studies of the infectivity and stability of extracts and homogenates derived from scrapie affected mouse brains. *J Comp Pathol* **1969**;79:101–8.
19. Hunter GD. The enigma of the scrapie agent: biochemical approaches

- and the involvement of membranes and nucleic acids. In: Prusiner SB, Hadlow W, eds. *Slow transmissible diseases of the nervous system*. Vol. 2. New York: Academic Press, 1979:365–86.
20. Prusiner SB, McKinley MP, Groth DF, et al. Scrapie agent contains a hydrophobic protein. *Proc Natl Acad Sci USA* 1981; 78:6675–9.
 21. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. *Cell* 1983; 35:57–62.
 22. Oesch B, Jensen M, Nilsson P, Fogh J. Properties of the scrapie prion protein: quantitative analysis of protease resistance. *Biochemistry* 1994; 33:5926–31.
 23. Bolton DC, McKinley MP, Prusiner SB. Molecular characteristics of the major scrapie prion protein. *Biochemistry* 1984; 23:5898–906.
 24. Lin X, Lee CG, Casale ES, Shih JCH. Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain PWD-1. *Appl Environ Microbiol* 1992; 58:3271–5.
 25. Wang JJ, Shih J. Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and a recombinant *B. subtilis* FDB-29. *J Ind Microbiol Biotechnol* 1999; 22:608–16.
 26. Wells GA, Hancock RD, Cooley WA, Richards MS, Higgins RJ, David GP. Bovine spongiform encephalopathy: diagnostic significance of vacuolar changes in selected nuclei of the medulla oblongata. *Vet Rec* 1989; 125:521–4.
 27. VanKeulen LJM, Schreuder BEC, Meloen RH, et al. Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. *Vet Pathol* 1995; 32:299–308.
 28. Schaller O, Fatzer R, Stack M, et al. Validation of a Western immunoblotting procedure for bovine PrP^{Sc} detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). *Acta Neuropathol* 1999; 98:437–43.
 29. Price NC, Johnson CM. Proteinases as probes of conformation of soluble proteins. In: Beynon RJ, Bond JS eds. *Proteolytic enzymes: a practical approach*, Oxford: IRL Press, 1989:163–80.
 30. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
 31. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Biotechnology* 1979; 24:145–9.
 32. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 1981; 112:195–203.
 33. Moynagh J, Schimmel H. Tests for BSE evaluated. *Nature* 1999; 400: 105.
 34. Rourke KI, Baszler TV, Besser TE, et al. Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue. *J Clin Microbiol* 2000; 38:3254–9.
 35. Krasemann S, Jürgens T, Bodemer W. Generation of monoclonal antibodies against prion proteins with an unconventional nucleic acid-based immunization strategy. *J Biotechnol* 1999; 73:119–29.
 36. VanKeulen LJ, Schreuder BE, Meloen RH, et al. Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. *Vet Pathol* 1995; 32:299–308.
 37. Korth C, Stierli B, Streit P, et al. Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody. *Nature* 1997; 390:74–7.
 38. Schimmel H, Catalani P, leGuern L, et al. Report: the evaluation of five rapid tests for the diagnosis of transmissible spongiform encephalopathy in bovines (2nd study). Brussels: Scientific Steering Committee, 2002 (available at: heinz.schimmel@irimm.jrc.be).
 39. Deslys JP, Comoy E, Hawkins S, et al. Screening slaughtered cattle for BSE. *Nature* 2001; 409:476–8.
 40. Fraser H, Bruce ME, Chree A, McConnell I, Wells GAJ. Transmission of bovine spongiform encephalopathy and scrapie to mice. *J Gen Virol* 1992; 73:1891–7.
 41. Taylor DM, Woodgate SL, Atkinson MJ. Inactivation of the bovine spongiform encephalopathy agent by rendering procedures. *Vet Rec* 1995; 137:605–10.
 42. Stolze A, Weber A, Reifenberg K, Laude H, Vilotte JL, Groschup MH. Bovine and ovine prion protein (PrP) transgenic mice as sensitive tools for the detection of BSE and scrapie infectivity [abstract P4.16]. In: Program and abstracts of the International Conference on Transmissible Spongiform Encephalopathies (Edinburgh) 2002.
 43. Safar JG, Scott M, Monaghan J, et al. Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice. *Nat Biotechnol* 2002; 20:1147–50.
 44. Wille H, Prusiner SB, Cohen FE. Scrapie infectivity is independent of amyloid staining properties of the N-terminally truncated prion protein. *J Struct Biol* 2000; 130:323–38.
 45. Bolton DC, McKinley MP, Prusiner SB. Identification of a protein that purifies with the scrapie prion. *Science* 1982; 218:1309–11.
 46. Oesch B, Westaway D, Wälchli M, et al. A cellular gene encodes scrapie PrP 27–30 protein. *Cell* 1985; 40:735–46.
 47. Dormont D. Agents that cause transmissible subacute spongiform encephalopathies. *Biomed Pharmacother* 1999; 53:3–8.
 48. Flechsig E, Hegyi I, Schwarz P, Collinge J, Weissmann C. Transmission of scrapie by steel-surface-bound prions. *Mol Med* 2001; 7:679–84.

ATTACHMENT 2

“Food Safety and Inspection Service (FSIS) Current Thinking on Measures that Could be Implemented to Minimize Human Exposure to Materials that Could Potentially Contain the Bovine Spongiform Encephalopathy Agent” – Pages 1 & 17.

January 15, 2002

**FOOD SAFETY AND INSPECTION SERVICE (FSIS)
CURRENT THINKING ON MEASURES THAT COULD BE IMPLEMENTED
TO MINIMIZE HUMAN EXPOSURE TO MATERIALS THAT COULD
POTENTIALLY CONTAIN THE
BOVINE SPONGIFORM ENCEPHALOPATHY AGENT**

FSIS is considering implementing a number of measures to minimize human exposure to materials that could potentially contain the agent that causes Bovine Spongiform Encephalopathy (BSE). Scientific and epidemiological studies have linked variant Creutzfeldt-Jakob Disease (vCJD), a chronic and fatal neurodegenerative disease that affects humans, to the consumption of beef products contaminated with the BSE agent. Neither vCJD nor BSE has been detected in the U.S. and the recently released Harvard Risk Assessment on BSE finds that, owing to already ongoing Federal programs, the U.S. is highly resistant to the introduction and spread of BSE in the U.S. cattle herd. However, FSIS believes that additional measures should be considered to minimize human exposure to BSE agents in the unlikely event that it is introduced in the U.S. This paper provides FSIS's thinking on policy options currently under consideration. FSIS requests public comment on the options discussed in this paper. Comments may be submitted to the FSIS Docket Room, Room 102, 300 12th Street SW, Washington, DC 20250-3700 and should be marked "FSIS current thinking on BSE." Copies of the Harvard Risk Assessment and this paper are available for viewing or copying in the FSIS Docket Room and on the Internet at: <http://www.fsis.usda.gov/oa/topics/bse.htm>.

Background

Bovine Spongiform Encephalopathy and Variant Creutzfeldt-Jakob Disease

BSE, commonly referred to as "Mad Cow Disease," is a slowly progressive degenerative disease that affects the central nervous system (CNS) of adult cattle. The typical incubation period (the time from when an animal becomes infected until it first shows disease signs) for BSE is from two to eight years. Following the onset of clinical signs, the animal's condition deteriorates until it either dies or is destroyed. This process usually takes from two weeks to six months. BSE is so named because of the spongy appearance of the brain tissue of infected cattle when sections are examined under a microscope.

BSE belongs to the family of diseases known as the transmissible spongiform encephalopathies (TSE's). Other TSE's include scrapie in sheep and goats, transmissible mink encephalopathy, feline spongiform encephalopathy, chronic wasting disease (CWD) in deer and elk, and in humans, kuru, classic Creutzfeldt-Jakob Disease (CJD), Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia, and variant Creutzfeldt-Jakob Disease (vCJD). The agent that causes BSE and other TSE's has yet to be fully characterized. There are three main theories on the nature of the agent: (1) the agent is a virus with unusual characteristics; (2) the agent is a prion—an abnormal form of a normal protein known as cellular prion protein; and (3) the agent is a virino—an "incomplete" virus composed of nucleic acid protected by host proteins. The agent is

the CNS of deer and elk. CWD is endemic in wild deer and elk populations in certain areas of the western U.S. and has also been identified in domesticated elk in six western states. Although there also is no epidemiological evidence of transmission of CWD to humans, at least one published study has reported that abnormal CWD prion proteins *in vitro* can convert normal human prion proteins into abnormal forms, albeit inefficiently.

Although deer and elk are not required to be slaughtered under Federal inspection, the facilities in which these animals are slaughtered and processed may be used to slaughter and process livestock that are subject to mandatory Federal inspection (i.e., cattle, sheep, swine, goats, horses, mules, and other equines). If deer or elk that contain the CWD agent are slaughtered in a facility that also slaughters livestock subject to mandatory Federal inspection, the equipment in the facility could be contaminated with the CWD agent, thereby potentially spreading the agent to federally inspected cattle and carcasses intended for human food.

FSIS believes that it would be prudent to develop methods that could prevent the CWD agent from contaminating equipment used to slaughter livestock subject to mandatory Federal inspection. The agents that cause BSE, CWD, and other TSE's are highly resistant to heat, ultra violet light, ionizing radiation, and common disinfectants and FSIS is not aware of any practical methods that could be used to sanitize equipment that has been contaminated with a TSE agent. Certain sanitation measures, such as treatment with sodium hypochlorite (20,000 ppm of available chlorine) and treatment with hot 2N sodium hydroxide solution for one hour, appear to be effective. But these sanitation methods are harsh on equipment and can be hazardous. FSIS is interested in receiving any scientifically based information on this subject.

Additional risk assessment modeling to be performed by Harvard

As mentioned above, the Harvard risk assessment identifies the pathways by which humans could potentially be exposed to the BSE agent from beef products. It does not provide an assessment of the possible reduction in human exposure that may be associated with the policy options contained in this paper. Therefore, FSIS has requested that Harvard assist FSIS in using the risk assessment model to evaluate the policy options discussed in this paper and alternatives. FSIS will use the results of the additional modeling to refine the policy options under consideration, to determine the effectiveness of measures in reducing the risk of potential exposure to the BSE agent, and to evaluate the benefits of various alternatives.

The measures that FSIS will evaluate with assistance from Harvard include:

- Prohibiting the use of bovine intestine for human food.
- Prohibiting the use of vertebral column as a source material in meat or meat food product recovery systems that use pressure to separate beef meat or beef meat food product from bone for cattle older than 24 months.

ATTACHMENT 3

**“Development of an Enzymatic Rendering Process for Prion-free
Animal Products” USDA-IFAFS Critical and Emerging Issues Grant.**

September 15, 2001



Current Research Information System **CRIS**

Item No. 1 of 1

ACCESSION NO: 0190574 **SUBFILE:** CRIS
PROJ NO: NC09120 **AGENCY:** CSREES NC.
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** EXTENDED
CONTRACT/GRANT/AGREEMENT NO: 2001-52100-11503 **PROPOSAL NO:** 2001-05313
START: 15 SEP 2001 **TERM:** 30 SEP 2003 **GRANT YR:** 2001
GRANT AMT: \$140,000

INVESTIGATOR: Shih, J. C.

PERFORMING INSTITUTION:
POULTRY SCIENCE
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RALEIGH, NORTH CAROLINA 27695

DEVELOPMENT OF AN ENZYMATIC RENDERING PROCESS FOR PRION-FREE ANIMAL PRODUCTS

NON-TECHNICAL SUMMARY: Mad cow disease has caused the death of 200,000 cattle in Europe. A new variant human Creutzfeldt-Jakob disease (CJD) may be caused by the consumption of infected or contaminated beef. It is imperative to develop a preventive measure to stop the disease in Europe and America. The degradation of prion protein, the causative agent of mad cow disease, by an enzyme called keratinase was discovered. This project will study and develop a good rendering process that uses the keratinase to destroy prions in animal products and stop the spread of the disease.

OBJECTIVES: The long-term goal of this project is to develop a "Good Rendering Process (GRP)" that uses PWD-1 keratinase to decompose infectious prion protein and consequently renders animal products bovine spongiform encephalopathy(BSE)-free. Work will be carried out collaboratively with ID-Lelystad in the Netherlands and BioResource International, Inc. (BRI) in Raleigh. In this project, the specific objectives are: 1) Production and purification of PWD-1 keratinase. 2) Enzymatic degradation of BSE and scrapie prions. 3) Development of methods of prion assay. 4) Strain development for keratinase production.

APPROACH: 1) Production and purification of PWD-1 keratinase: Keratinase will be produced at NCSU by fermentation in 15-L and 150-L fermentors. Purification will be carried out by ultrafiltration and CM-cellulose and Sepadex chromatographies. 2) Enzymatic degradation of BSE and scrapie prions: Conditions for the in vitro degradation of the prion protein by keratinase will be studied at ID-Lelystad. Optimization of variable parameters will include pretreatment, keratinase levels, digestion time and temperature, other proteases, additives, etc. 3) Development of methods of prion assay: ID-Lelystad will test a broad set of antibodies against prion and a broad range methods, including immunohistochemistry, Western blotting, dot blotting, indirect ELISA, etc. 4) Strain development for keratinase production: BRI will carry out genetic modification of *Bacillus licheniformis* strains to over express the keratinase gene. Multiple copies of keratinase

gene will be integrated onto the chromosome for stable hyper-production of the enzyme. Special vectors will be constructed and special host cells will be used for protoplast transformation, followed by screening and selection to accomplish this objective.

PROGRESS: 2001/10 TO 2002/09

Infectious prion protein is believed to be the causal agent of mad cow disease, or bovine spongiform encephalopathy (BSE). A bacterial keratinase discovered in this laboratory was tested for its degradative activity on the prion protein and detection was done with routine diagnostic method. It was found that a combination of pre-heating and enzymatic digestion on BSE brain tissue, the prion protein was broken down to the undetectable level. This discovery indicates a potential enzymatic process for prion inactivation and prevention of the disease. Evaluation of the process in disinfection of prions in animals is in progress.

IMPACT: 2001/10 TO 2002/09

Prevention of mad cow disease is of utmost importance. The disease has a strong impact not only on human health, but also on the multi-billion dollar beef industry and the economy associated with the industry.

PUBLICATIONS: 2001/10 TO 2002/09

Longeveld, J.P.M., Wang, J.J., Shih, G.C., Garssen, G.J., Van de Wiel, D.F.M., Bossers, A. and Shih, J.C.H. (2003) Enzymatic degradation of prion protein from infected cattle and sheep brain stem. EMBO J. (submitted)

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