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Prion inactivation by the Maillard reaction

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Abstract

Since variant Creutzfeldt-Jakob disease (vCJD) has been suspected to be attributable to the infectious agents associated with bovine spongiform encephalopathy (BSE), it is important to prevent the transmission of pathogenic forms of prion protein (PrP^{Sc}) through contaminated feeding materials such as meat and bone meal (MBM). Here, we demonstrate that the Maillard reaction employing a formulation of glucose in combination with sodium hydrogen carbonates effectively reduced the infectivity (approximately 5.9-log reduction) of a scrapie-infected hamster brain homogenate. In addition to a bioassay, a protein misfolding cyclic amplification (PMCA) technique, in which PrP^{Sc} can be amplified *in vitro*, was used as a rapid test for assessing PrP^{Sc} inactivation. The PMCA analysis also indicated that the PrP^{Sc} level in the infected material significantly decreased following the Maillard reaction. Therefore, the Maillard reaction can be employed for the decontamination of large amounts of byproducts such as MBM.

Keywords: Prion inactivation; Meat and bone meal; Maillard reaction; Bioassay; Protein misfolding cyclic amplification

Prion diseases or transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders that have been described in humans as Creutzfeldt-Jakob disease (CJD), in sheep and goats as scrapie, and in cattle as bovine spongiform encephalopathy (BSE) [1]. A characteristic of TSE is the accumulation of a protease-resistant, misfolded prion protein (PrP^{Sc}), which is a pathogenic variant of the host-encoded prion protein (PrP^{C}) [2,3]. Since PrP^{Sc} retains its infectivity after undergoing routine sterilizing processes, harsh physical or chemical sterilization procedures are required for its inactivation [4,5].

BSE is an emerging disease that first appeared in the United Kingdom after 1986 [6]. It appears that the cause of the BSE outbreak was feeding PrP^{Sc}-contaminated meat and bone meal (MBM) acquired from rendered carcasses of BSE- or scrapie-infected ruminants to healthy cattle [7,8]. It is thought that the recycling of BSE-infected bovine tissues augmented the concentration of PrP^{Sc} in commercial MBM, thus causing the subsequent BSE epidemics [9]. Thus, in order to ensure that byproducts such as MBM can be safely used in the future, the development of a technology that can be used for treating large amounts of byproducts at low costs is required.

In the present study, we report a novel method for prion inactivation by chemical modification of proteins based on the principle of the Maillard reaction [10–13]. The Maillard reaction is a very complicated reaction between reducible carbohydrates and reactive amino acid residues of proteins such as lysine, arginine, and tryptophan [14–16]. The reaction products consist of a large variety, for example, brown protein polymers called "melanoidins." Since the Maillard reaction irreversibly modifies the proteins, the structural changes in PrP^{Sc} induced by the Maillard reaction are expected to be effective for reducing the infectivity. We therefore investigated the efficacy of the Maillard reaction in scrapie prion inactivation by a bioassay that examined

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the remnant infectivity in the treated samples. In addition, a protein misfolding cyclic amplification (PMCA), which is a highly sensitive method for detecting minute amounts of PrP^{Sc} [17,18], was employed for the detection of residual PrP^{Sc} following the Maillard reaction.

Materials and methods

Maillard reaction. The hamster-adapted scrapie prion strain Sc237 was propagated in hamsters. The brains of hamsters in the terminal stage of the disease, titrating $5 \times 10^{8.5}$ lethal dose (LD_{50}) per gram by bioassay [19], were pooled and homogenized at a concentration of 20% (w/v) in PBS. The homogenate (250 μl) was mixed with an equal volume of 0%, 10%, 20% or 40% (w/v) of glucose–PBS solution in the presence of 2% (w/v) sodium hydrogen carbonate. Since the kinetics of the Maillard reaction depend upon the pH and temperature conditions and the maximum reaction velocity can be obtained in pH ranges of 9–10 [20–22], sodium hydrogen carbonate was added to the reaction buffer as a pH-controlling reagent. After incubation for 30 min at room temperature, the samples were heated to 100 °C and kept for 3 h. After the above treatment, the samples were cooled down to the ambient temperature and the resultant materials were stored at −80 °C until further use.

Western blotting and sequential PMCA. The samples treated by the Maillard reaction and a control sample were mixed with an equal volume of 2× SDS sample buffer and incubated at 100 °C for 5 min. The samples were separated by SDS–PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking, the membrane was incubated for 1 h with horseradish peroxidase-conjugated 3F4 (1/2500; Signet Laboratories, Dedham, MA) or SAF32 (1/2500; Cayman chemical, Ann Arbor, MI) monoclonal antibodies. After washing, the blotted membrane was developed with the ECL + Plus Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, England) according to the manufacturer's instructions. The chemiluminescence signals were analyzed using the Light Capture System (ATTO, Tokyo, Japan).

We used the PMCA procedure described in our previous study [19]. Briefly, normal hamster brains were homogenized at 10% (w/v) in PBS-containing complete protease inhibitors (Roche Diagnostics, Mannheim, Germany), 1% Triton X-100, and 4 mM EDTA. The supernatant was separated by brief centrifugation and used as the PrP^{C} source. The sample was diluted 1:10 in normal brain homogenate, and one round of the PMCA reaction was carried out by performing 40 cycles of sonication followed by incubation at 37 °C for 1 h. Next, the process of dilution (1:10) of the PMCA product and its subsequent amplification was repeated two times. In the case of PMCA products, before and after each round of amplification, samples (10 μl) were mixed with 10 μl of proteinase K (PK) solution (100 μg/ml) and incubated at 37 °C for 1 h. The digested materials were analyzed by Western blotting using the 3F4 antibody as described above.

Bioassay. The inactivated samples were ultrasonicated immediately before inoculation. In the preliminary experiments, convulsions developed in the mice inoculated with the samples containing 5% or 10% glucose at final concentration, and some mice died within 15 min. Therefore, the samples with 20% glucose at final concentration and those without glucose (temperature control) were injected intracerebrally into five or six Tg52NSE mice (20 μl per mouse) that exhibited over expression of hamster PrP^{C} in their nerve system [23]. All animal experiments were performed according to the guidelines of the National Institute of Animal Health.

Results

Characterization of PrP^{Sc} after the Maillard reaction

Fig. 1 illustrates the results of Western blotting following the Maillard reaction. The signal intensities of both

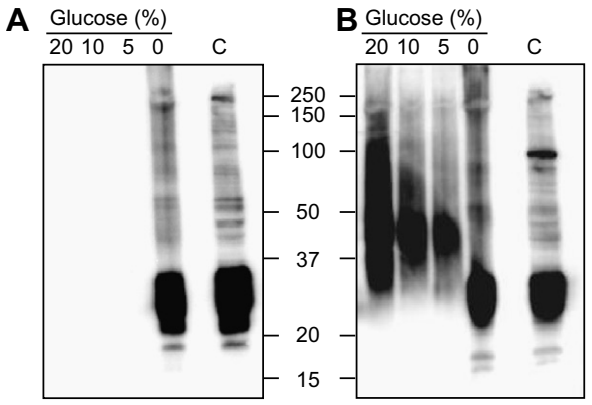

Fig. 1. Western blot analysis of prion protein in the Sc237-infected hamster brain homogenate following the Maillard reaction. A 20% brain homogenate and 0–40% of the glucose solution were mixed in equal quantities: the mixture was then incubated at 100 °C for 3 h in the presence of 2% sodium hydrogen carbonate. The samples were separated by SDS–PAGE, and the blotted membranes were then incubated with 3F4 (A) or SAF32 (B) antibodies. The lanes labeled "C" were the controls in which no glucose was added to the mixture before heating. The positions of molecular-weight standards (15–250 kDa) are also shown.

3F4 (A) and SAF32 (B) epitopes of prion protein did not differ greatly in the samples before (lane "C") and after heating in the presence of sodium hydrogen carbonate alone. The 3F4 epitope (MKHM) could no longer be detected following the Maillard reaction in the presence of 5–20% of glucose. The chemical modification of the lysine residue located in the epitope by glucose is probably responsible for this observation. On the other hand, the SAF32 epitope within the octapeptide repeat region (WGQPHGGG) could be detected as broad signals following the Maillard reaction. The epitope did not consist of reactive amino acid residues; therefore, the antigenicity was maintained after the Maillard reaction. The molecular weight of the prion protein detected by the SAF32 antibody was significantly increased, indicating that the addition of glucose to the reactive amino acid residues located outside of the epitope proceeded to various extents during the reaction. These results suggested that the molecular structure of prion protein was considerably altered by the Maillard reaction.

Assessment by the bioassay and PMCA

The control mice inoculated with the untreated samples developed the disease after an average period of 45 ± 2 days (average ± SD, $n = 5$, Fig. 2). The mice inoculated with the heat-treated (100 °C for 3 h) control sample died after an average period of 49 ± 2 days ($n = 5$). The onset of the disease in the mice inoculated with the sample containing 20% glucose at final concentration treated by the Maillard reaction was significantly delayed; however, they died after an average period of 115 ± 30 days ($n = 6$). The reduction in infectivity was estimated to be

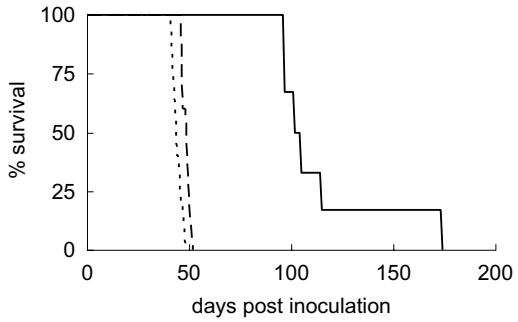

Fig. 2. Survival curves of Tg52NSE mice inoculated with the infected brain homogenates. Dotted line, no treatment; broken line, heat treatment (100 °C, 3 h) alone; solid line, the Maillard reaction with 20% glucose at final concentration.

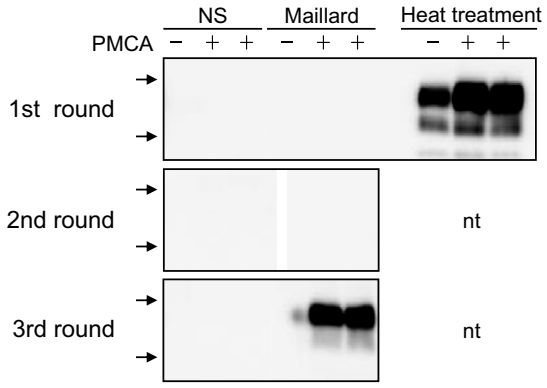

Fig. 3. PMCA of the sample containing 20% glucose at final concentration following the Maillard reaction. The sequential PMCA was performed according to the procedure described in our previous report [19]. The samples were analyzed before (−) and after (+, in duplicate) each round of amplification by Western blotting following digestion with PK. The lanes labeled "NS" were the controls in which the uninfected brain homogenate (PrP^C only) was treated in the same manner. No signals were detected in these samples. The lanes labeled "Heat treatment" denote the controls in which the infected brain homogenate was subjected to only heat treatment (100 °C, 3 h). The arrows indicate the positions of the molecular-weight markers corresponding to 30 and 20 kDa. nt, not tested.

$10^{-5.9}$ on the basis of the incubation time obtained from the previous study [19].

Fig. 3 shows the results from the amplification of the samples treated by the Maillard reaction. In the first and second rounds of amplification, no protease-resistant PrP (PrP^{res}) signals were detected in the samples. However, after three rounds of amplification, the PrP^{res} signals could be detected in both the duplicated samples. These results suggested that a minute amount of PrP^{Sc} remained unmodified in the sample treated by the Maillard reaction.

Discussion

The Maillard reaction has been considered to be the chemical reaction responsible for the browning and the loss of nutritive value of foods. The reaction involves the formation of CO_2 from the carboxyl group of the amino acid residue of proteins and the development of brown pigments (melanoidins), which might be nitrogen-containing polymeric substances of proteins. There are numerous reports describing the chemistry of this complex reaction to identify its various pathways, including reaction parameters such as pH, temperature, time, sugar reactivity, concentration of the reagents, water content, and glass transition temperature [21,22,24]. In the present study, we assessed various concentrations of glucose in the presence of sodium hydrogen carbonate for the ability to reduce infectivity and decrease PrP^{Sc} levels by the bioassay and the PMCA, respectively.

Since PrP^{Sc} is resistant against physicochemical inactivation procedures, very harsh treatments are required for complete inactivation of PrP^{Sc}. A majority of chemicals, that are strong protein-modification agents may cause secondary damage such as environmental pollution and are not suitable for MBM treatment. On the other hand, the advantages of the Maillard reaction are the low corrosive impact and chemical toxicity. The reactants are nontoxic reducible carbohydrates such as glucose; further, the products appear to be harmless because the Maillard reaction generally occurs during the manufacturing processes of heat-treated foodstuffs.

In conclusion, the present study demonstrated that the Maillard reaction induced a strong modification of PrP^{Sc} resulting in an effective reduction of the infectivity. Since PMCA was capable of amplifying unmodified PrP^{Sc} following the Maillard reaction and three rounds of PMCA required only 6 days, the PMCA technique would be very useful for further improvements in the process of prion decontamination by using the Maillard reaction. Studies are now in progress to validate glucose formulation as an effective yet routinely applicable reprocessing procedure for prion decontamination.

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