Characterization and enzymatic degradation of Sup35NM, a yeast prion-like protein

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Abstract

Transmissible spongiform encephalopathies (TSEs) are believed to be caused by an unconventional infectious agent, the prion protein. The pathogenic and infectious form of prion protein, PrP^{Sc}, is able to aggregate and form amyloid fibrils, very stable and resistant to most disinfecting processes and common proteases. Under specific conditions, PrP^{Sc} in bovine spongiform encephalopathy (BSE) brain tissue was found degradable by a bacterial keratinase and some other proteases. Since this disease-causing prion is infectious and dangerous to work with, a model or surrogate protein that is safe is needed for the in vitro degradation study. Here a nonpathogenic yeast prion-like protein, Sup35NM, cloned and overexpressed in *E. coli*, was purified and characterized for this purpose. Aggregation and deaggregation of Sup35NM were examined by electron microscopy, gel electrophoresis, Congo red binding, fluorescence, and Western blotting. The degradation of Sup35NM aggregates by keratinase and proteinase K under various conditions was studied and compared. These results will be of value in understanding the mechanism and optimization of the degradation process.

Keywords: BSE; prion; PrP^{Sc}; Sup35NM; yeast prion; prion surrogate protein; enzymatic degradation

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a unique group of fatal neurodegenerative disorders found in humans and animals (Prusiner et al. 1998). They include diseases such as kuru and Creutzfeld-Jakob disease (CJD) in humans, scrapie in sheep, bovine spongiform encephalopathy (BSE, widely known as mad cow disease) in cattle (Parchi and Gambetti 1995), and TSEs in other mammals (Burger and Hartsough 1965; Williams and Young 1980; Leggett et al. 1990). All of these diseases are characterized by extensive neuronal loss giving brain tissue a spongy appearance.

The pathological isoform of prion protein (PrP^{Sc}) aggregates in diseased tissues and, in common conditions, is resistant to digestion by proteases (Caughey et al. 1991; Pan et al. 1993). It was recently found that PrP^{Sc} in diseased tissues can be degraded by a feather-degrading keratinase and other microbial proteases when the tissue homogenate was preheated at 115°C (Langeveld et al. 2003). Independently, mouse bioassays showed a significant reduction of infectivity after a proteolytic inactivation process (McLeod et al. 2004). These results indicated a potential method of enzymatic inactivation of prion protein.

PrP^{Sc} is pathogenic and dangerous to handle. A mutant recombinant prion produced in *Escherichia coli* without glycosylation was recently found to be able to induce neurologic dysfunction (Legname et al. 2004). Experimentation with prion tissues must be strictly controlled and is inordinately expensive. A nonpathogenic prion surrogate protein which has similar physical-chemical properties

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will be useful for research in this field. In addition, it may be used as a marker to test or screen putative prion inactivation methods.

The yeast with prion-like proteins was once thought to be useful as an *in vivo* model to screen for anti-prion drugs (Saupe 2003). Sup35p from *Saccharomyces cerevisiae* is a translation termination factor that can convert into an insoluble aggregate, a property similar to prion protein (Masison and Wickner 1995; Paushkin et al. 1996). The structure of Sup35p can be divided into three regions, namely, N, M, and C, based on their positions and different functions. Individual regions and combinations have been cloned, expressed, and purified from *E. coli*. They were characterized for their properties to form aggregates, or amyloid, *in vitro* (Derkatch et al. 1997; Glover et al. 1997; King et al. 1997; Osherovich et al. 2004). The aggregates of Sup35N and Sup35NM were found to be proteinase K-resistant (Kushnirov et al. 2000).

For these reasons, Sup35NM was selected for the present study of production, purification, and characterization. Its degradation by keratinase and proteinase K under various conditions was also studied and compared. Being nonpathogenic, Sup35NM could be a candidate for a safe prion surrogate protein.

Results

Properties of Sup35NM

Sup35NM was cloned, produced, and purified from *E. coli*. When allowed to aggregate, Sup35NM formed amyloid fibrils (Fig. 1A). After heating to 100°C for 10 min, the fibrils disappeared (Fig. 1B).

Congo red binding was used to measure the aggregation under two different conditions: slow rotating (5 rpm) at room temperature without seeding, and seeding with 2% preformed Sup35NM amyloid to form aggregates without rotating. Congo red binding reached a plateau in 5 h for both samples (Fig. 2A). However, SDS-PAGE and Western blotting analyses showed that the samples were not fully aggregated, with soluble monomeric Sup35NM still

Figure 1. Electron micrographs of negatively stained Sup35NM amyloid fibrils (A) and after heating at 100°C for 10 min (B).

Figure 2. Time course of Sup35NM aggregation at 25°C over a 48-h time course as monitored by Congo red binding, SDS-PAGE, and Western blot. (A) Congo red binding assay. Congo red only (▲); rotating with 5 rpm (●); seeding with 2% preformed fibrils (■). At various times, duplicate aliquots were diluted to 2 μ M protein and incubated together with 10 μ M Congo red. (B) SDS-PAGE analysis of samples with Coomassie blue with 5 rpm rotating over time. (C) Western blots of the same samples probed by anti-M antibody. Aliquots were not heated (–) except for the last lane, which was heated (+) at 100°C for 10 min before the analysis. Mono, monomeric Sup35NM; Poly, Sup35NM amyloid fibrils.

detectable after 5 h. At 22–48 h, soluble protein was almost gone. Heating the same 48-h sample at 100°C for 10 min produced a band with the same mobility and intensity as those of the band at 0-time (Fig. 2B,C). Insoluble aggregates that stuck in the loading wells of the gel did not stain well by Coomassie blue (Fig. 2B), but they could be transferred onto PVDF membrane by Western blotting and detected by immunostaining (Fig. 2C). This result also indicated that Congo red binding is not a good indicator for the aggregation of Sup35NM. Western blotting clearly showed a gradual increase of amyloid Sup35NM as the insoluble protein trapped in the loading wells. After heating the 48-h sample at 100°C for 10 min, insoluble amyloid disappeared and the soluble Sup35NM reappeared (Fig. 2B,C). As observed with electron microscopy (Fig. 1B), deaggregation of fibrillar Sup35NM resulted from heating at 100°C for 10 min.

Congo red did not distinguish the aggregation after 5 h (Fig. 2A). However, the binding of Thioflavin T (ThT), as measured by fluorescence emission, clearly showed that unseeded Sup35NM took 24–48 h of slow rotation to complete aggregation (Fig. 3A). Samples at each time point also were analyzed by SDS-PAGE (Fig. 3B). Along with aggregation time, fluorescence emission increased and monomeric Sup35NM in SDS-PAGE decreased. The comb of loading wells is not shown in Figure 3B, as in Figure 2B. The insoluble fibrils in the wells were not stained by Coomassie blue.

Enzymatic degradation

Sup35NM monomers were readily degraded by keratinase and proteinase K (data not shown), whereas the Sup35NM aggregates were more resistant to both enzymes. Degradation by both enzymes was compared at different levels of enzyme units under the same conditions (Fig. 4). SDS-PAGE analysis indicated complete degradation at the high enzyme level (7.3 U) and incomplete degradation at lower enzyme levels, where two different enzymes produced different patterns of protein fragments (Fig. 4A,D). Western blotting probed with anti-M serum (Fig. 4B,E) and anti-N serum (Fig. 4C,F) clearly showed that the high enzyme level (7.3 U) resulted in more degradation.

Preheating and digestion temperatures

Preheating of BSE homogenates followed by keratinase digestion effectively renders prion proteins undetectable in an *in vitro* assay (Langeveld et al. 2003). To determine

Figure 3. Time course of Sup35NM aggregation as measured by fluorescence emission of ThT at 25°C over 48 h. (A) Fluorescence emission of Thioflavin T (ThT) assay. Amyloid Sup35NM (■); Sup35NM with 5 rpm rotating (●). At various times, aliquots of the reactions were diluted to 0.2 μ M protein and incubated together with 20 μ M ThT. (B) SDS-PAGE stained with Coomassie blue. Aliquots of the reactions at various times were added with sample buffer, then loaded onto the gel without heating. Aliquot samples were not heated (–) except for the last lane, which was heated (+) at 100°C for 10 min.

Figure 4. The degradation of Sup35NM amyloid fiber by proteinase K (PK) and keratinase (KE). Each reaction contained 50 μ g of protein with different enzyme amounts, 1.5 U (+) or 7.3 U (++), and was incubated at 37°C for 15 min. (A,D) SDS-PAGE stained with Coomassie blue. (B,E) Western blots probed by anti-M antibody. (C,F) Western blots probed by anti-N antibody. Std, 0.75 μ g of untreated, purified Sup35NM.

whether preheating treatment has a similar effect on the keratinase digestion of amyloid Sup35NM, three different preheating temperatures (80°, 100°, and 115°C) for 10 min were tested. We used the amyloid Sup35NM heated at 115°C alone without enzyme as a control. The preheated samples were then treated with keratinase at 37° or 50°C. The resulting digestions were analyzed by SDS-PAGE and Western blotting (Fig. 5). For 37°C digestion (Fig. 5A,C,E), preheating at 115° and 100°C made the amyloid degradable. After preheating at 80°C, or no preheating, the amyloid was resistant to the digestion when the enzyme level was 1.5 U and became less resistant when the enzyme level was increased to 7.3 U (Fig. 5A,C,E). For 50°C digestion, after preheating at 80°C or no preheating, again the amyloid was resistant to digestion at the low enzyme level (1.5 U). However, at the high enzyme level (7.3 U), the amyloid, regardless of whether the sample was preheated, was degradable (Fig. 5B,D,F).

Digestion time at different keratinase levels

As we did previously (Langeveld et al. 2003), preheating was carried out at 115°C for 10 min, and the digestion conditions for keratinase were determined for the enzyme level and digestion time. With and without

Figure 5. The effect of preheating and digestion temperatures on the degradation of Sup35NM aggregates by keratinase. Each reaction contained 50 μ g of amyloid protein, was preheated, and digested by different enzyme amounts: 1.5 U (+) or 7.3 U (++) at 37°C (A,C,E) or 50°C (B,D,F) for 30 min. (A,B) SDS-PAGE stained with Coomassie blue. (C,D) Western blots probed by anti-M antibody. (E,F) Western blots probed by anti-N antibody. Samples were preheated at 115°, 100°, or 80°C for 10 min. Std, 0.75 μ g of untreated, purified Sup35NM; Mono, monomeric Sup35NM; Poly, Sup35NM amyloid fibrils.

preheating, monomeric and amyloid Sup35NM were treated with two levels of keratinase, 1.5 U and 7.3 U, at 50°C for three digestion times, 15, 30, and 60 min. At the low enzyme level, preheated Sup35NM amyloid was degraded faster than the monomers, while the amyloid form could be detected after 60 min (Fig. 6A,C,E). Digestion at the high enzyme level produced the same result. Preheating rendered Sup35NM amyloid readily degradable (Fig. 6B,D,F), the same as was found for the BSE prion protein (Langeveld et al. 2003).

Discussion

Prion-like proteins from yeast have been tested as an *in vivo* surrogate to screen anti-prion drugs (Saupe 2003) and as a model to study “species barriers” (King and Diaz-Avalos 2004; Tanaka et al. 2004). In the present study, Sup35NM was produced and purified. Its property of aggregating into polymer-like fibrils was

confirmed by electron microscopy (Fig. 1), and the different conditions for enzymatic degradation were tested.

The process of Sup35NM aggregation was analyzed by Congo red binding, fluorescence of ThT binding, SDS-PAGE, and Western blots. On a time course from 0 to 48 h, samples of aggregating Sup35NM were analyzed by SDS-PAGE. At the same time points, Congo red and ThT samples were also prepared and analyzed. A gradual increase in Congo red binding was found from 0 to 5 h before leveling off (Fig. 2). A gradual increase in ThT binding was detected during the course of 24 h before total saturation (Fig. 3). The agreement of increase of ThT fluorescence with the decrease of monomeric Sup35NM was confirmed by SDS-PAGE analysis (Fig. 3). The difference between the two spectrometric methods is believed to be due to the fact that Congo red binding detected changes in protein folding, specifically, the formation of β -sheets or the early stages of β -oligomer formation, as opposed to the larger

Figure 6. Time course of degradation. Amyloid Sup35NM, preheated at 115°C for 10 min, was digested by keratinase at 50°C for different reaction times. Each reaction contained 50 μ g of amyloid protein with 1.5 U (*A,C,E*) or 7.3 U (*B,D,F*) keratinase. (*A,B*) SDS-PAGE stained with Coomassie blue. (*C,D*) Western blots probed by anti-M antibody. (*E,F*) Western blots probed by anti-N antibody. Std, 0.75 μ g of untreated, purified Sup35NM; Mono, monomeric Sup35NM; Poly, Sup35NM amyloid fibrils.

aggregates of Sup35NM that ThT fluorescence detects (Baskakov et al. 2002). For this reason, ThT binding monitored by fluorescence emission should be the method of choice for monitoring the formation of amyloid fibrils.

In our previous study, PWD-1 keratinase and some other proteases were found to be able to degrade PrP^{Sc} in brain stem tissues from infected cattle and sheep (Langeveld et al. 2003). However, preheating treatment of the tissue homogenate is needed for complete digestion of PrP^{Sc} by these enzymes. It is possible that the preheating at 115°C caused a change in the structure of the PrP^{Sc} subjected to enzymatic degradation. In the present study with Sup35NM aggregates or fibrils, the same scenario was observed. Compared with its monomeric form, amyloid Sup35NM was found to be relatively resistant to both keratinase and proteinase K (Fig. 4).

The keratinase may be slightly more active than proteinase K in degrading polymeric Sup35NM when they were at the same level of enzyme units. More studies will be needed to determine the difference in activity of these two enzymes against prions. More importantly, both enzymes produced defined patterns of degradation products or fragments, and they are different (Fig. 4). This indicated that the cleavage sites of keratinase and proteinase K are specific and different. It will be of interest to identify these enzyme-specific cleavage sequences. The incomplete digestion can provide a characteristic marker of Sup35NM as the surrogate protein of prions.

In conclusion, various conditions to optimize partial and complete degradation of Sup35NM amyloid fibrils were studied (Figs. 5, 6). Ultimately, the most effective means will be verified with infected brain tissue and PrP^{Sc} and determined by infectivity tests in animals.

Sup35NM aggregates are less resistant to enzymatic degradation than PrP^{Sc} in BSE tissue, indicating the difference in structures. To fulfill the role of a safe, simple, and cost-effective surrogate protein for screening and optimizing experimental conditions, more comparative studies between Sup35NM and PrP are needed.

Since the discovery of enzymatic degradation of prion protein (Langeveld et al. 2003; McLeod et al. 2004), it will be of great interest to learn more about the interaction of the enzyme and prion protein and the mechanism of degradation. Further studies will focus on the protein intermediates produced from partial degradation to determine the cleavage sites in the Sup35NM amyloid structure. Also, it is possible that deaggregation and unfolding of the polymeric structure are the prerequisite steps, such as by preheating, for the proteolytic hydrolysis that follows. Being nonpathogenic and fairly easy to prepare, Sup35NM is a good candidate as a prion model for many research and technological applications. A similar protein molecule was recently developed and tested for its prion-like properties (Wang et al. 2005). Sup35NM and this molecule will be compared to determine the abilities of each to serve as a safe prion marker for disinfection processes and metabolic studies. In this study, we specifically evaluated Sup35NM properties.

Materials and methods

Materials

Isopropyl β -D-thiogalactoside (IPTG), sodium phosphate, potassium phosphate, azocasein, Congo red, and proteinase K were from Sigma Chemicals. Urea was from Promega. Thioflavin T was from Acros Organics. The Q Sepharose fast flow and the ECL Western blotting analysis system were from Amersham Pharmacia. The CHT ceramic hydroxyapatite, the PVDF membranes, and the anti-rabbit antibody conjugated to horseradish peroxidase were from Bio-Rad. The chemiluminescence reagent was from Pierce. Polyclonal antibodies produced by Biosource based on the epitopic peptide sequences, Ac-QGGYQQYNPDAGYQ-amide (Liu et al. 2002) and Ac-CAPKPKKTLKLVSSEG-amide (T.R. Serio, pers. comm.), were used to generate anti-Sup35NM polyclonal antisera against amino acids 55–68 (anti-N) and 135–148 (anti-M), respectively.

Keratinase was produced and purified as described (Lin et al. 1992, 1997).

Preparation of Sup35NM

The expression plasmid pJC25NMstop was a gift from Dr. Susan L. Lindquist (Whitehead Institute, Massachusetts Institute of Technology). Sup35NM was purified as described (Chernoff et al. 2002) with the following modification. Transformed colonies were inoculated into a 50-mL LB-ampicillin (50 μ g/mL) flask and incubated for 5 h at 37°C, and then 20 mL of the growing culture was removed and added into a fresh

500-mL LB-Amp (50 μ g/mL) medium. After induction with 1 mM IPTG at an A_{600} of ~ 0.6 , cells were harvested after 3 h of induction. Cell pellets were collected by centrifugation and stored at -80°C . Cells were lysed in 50 mL lysis buffer (10 mM Tris-HCl [pH 7.2], 1 mM DTT, 1 mM PMSF, 8 M urea) for 30 min at 25°C . The lysate was then cleared by centrifugation at 12,000g for 20 min at 10°C . The cleared supernatant was applied to a Q Sepharose column (2.5 $\times$ 7.5 cm) pre-equilibrated with lysis buffer. The column was washed with 5 $\times$ bed volumes of Q wash buffer I (10 mM Tris-HCl [pH 7.2], 1 mM DTT, 1 mM PMSF, 8 M urea, 85 mM NaCl), and 5 $\times$ volumes of Q wash buffer II (10 mM Tris-HCl [pH 7.2], 8 M urea, 150 mM NaCl). The protein was eluted in 3 volumes of Q elution buffer (10 mM Tris-HCl [pH 7.2], 8 M urea, 200 mM NaCl). The eluant from the Q Sepharose was then loaded directly onto a hydroxyapatite column (1.5 $\times$ 12 cm). The column was pre-equilibrated with Q elution buffer. After loading, the column was washed with 2 $\times$ volumes of HA wash buffer I (1 mM potassium phosphate [pH 6.8], 8 M urea, 1 M NaCl) and then with 2 $\times$ volumes of HA wash buffer II (25 mM potassium phosphate [pH 6.8], 8 M urea). The protein was eluted using a stepwise gradient of 75 mM and 125 mM potassium phosphate (pH 6.8) in 8 M urea and then with 2 $\times$ volumes of 125 mM potassium phosphate (pH 6.8) in 8 M urea. Fractions (5 mL) were analyzed by 15% SDS-PAGE (loading 10 μ L per lane). Protein concentrations were determined with the calculated extinction coefficient of 0.90 for a 1 mg/mL Sup35NM solution at 280 nm absorbance (Gill and von Hippel 1989). Anhydrous methanol (100%) was added to precipitate Sup35NM on ice at a ratio of 5:1. The supernatant was removed and the pellet was stored in 70% (v/v) methanol (1/2 volume of supernatant) at -80°C (Serio et al. 1999).

Aggregation and deaggregation of Sup35NM

Methanol-precipitated Sup35NM was resuspended in 25 mM potassium phosphate (pH 8.0). Sup35NM after sitting at 25°C for 3 d can aggregate into fibrils. These fibrils can be used as seeding material. Typically, 2% preformed fibrils were added to freshly suspended Sup35NM to accelerate aggregation. Alternatively, the resuspended Sup35NM in buffer was allowed to undergo a slow rotation at 5 rpm. Both methods were carried out at room temperature. For deaggregation, amyloid Sup35NM was boiled at 100°C for 10 min.

Electron microscopy

Sup35NM protein was negatively stained according to Chernoff et al. (2002). Here, 5 μ L of a 5 μ M protein solution was applied to a glow-discharged 400 mesh carbon-coated copper grid followed by staining with several drops of 2% (w/v) aqueous uranyl acetate, then air-dried. Samples were then observed in a JEOL-100S Transmission Electron Microscope at an accelerating voltage of 120 kV.

Congo red binding assay

Congo red binding assays were carried out as described previously (Klunk et al. 1989). Congo red was dissolved in Congo red binding buffer (CRBB) (5 mM potassium phosphate [pH 7.4], 150 mM NaCl) to give a concentration of 10 mM. Methanol-precipitated Sup35NM was resuspended in CRBB

to 10 μM . Samples were first heated at 100°C for 10 min to deaggregate the preformed fibrils, and reaggregation was started by incubation at 25°C. At indicated times, protein was diluted to 2 μM with 10 μM Congo red in CRBB and incubated for 30 min at 25°C before measuring. To calculate moles of Congo red bound/L of solution, the following equation was used: $\text{mol CR}_{\text{bound}}/\text{L} = (A_{540}/25295) - (A_{477}/46306)$.

Thioflavin T binding assay

The Thioflavin T (ThT) assay was carried out as described (Chernoff et al. 2002). A stock solution of 1 mM ThT in water was prepared fresh for each experiment. Methanol-precipitated Sup35NM was resuspended in CRBB to 10 μM . Samples were heated at 100°C for 10 min and then started to aggregate at 25°C as described above. At indicated times, Sup35NM protein was diluted to 0.2 μM in the presence of 20 μM ThT in CRBB. ThT fluorescence was monitored using a Quantech fluorometer (Barnstead Thermolyne), with excitation at 450 nm and emission at 481 nm (excitation slit, 1 nm; emission slit, 10 nm; 0.4-cm cuvette).

Azocasein assay

The keratinase and proteinase K activities were measured as described (Lin et al. 1992, 1997) with some modification. For a standard assay, 5 mg of azocasein was added with 0.8 mL of 50 mM potassium phosphate buffer (pH 7.5). Completely suspended azocasein solution was preheated at 37°C for 10 min. Five μL of appropriately diluted enzyme solution was added, and the mixture was incubated at 37°C for 10 min. For the control reaction, 5 μL of diluted buffer was added to azocasein solution. The reaction was terminated by the addition of 0.2 mL of 10% trichloroacetic acid (TCA) and put on ice for 10 min. Samples were centrifuged (14,000g, 4°C, 10 min), and the released azo-peptides were in the supernatant. The absorbance of the supernatant was measured with a spectrophotometer (Shimadzu Scientific Instruments). In this assay, we defined 1 U of enzyme activity as an increase in A_{450} of 0.01 after 10 min in reaction compared to the control.

Protease resistance of amyloid Sup35NM

This method was modified from a Sup35pN proteinase K resistance assay (King et al. 1997). About 50 μg of Sup35NM samples, monomeric or amyloid Sup35NM, were suspended in 25 μL of 25 mM potassium phosphate buffer (pH 8.0). Enzyme (2 μL) was added and the mixture was incubated for various periods of time at the regular assay temperature, 37°C, or at the keratinase optimum temperature, 50°C (Lin et al. 1992). Reactions were terminated by adding phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 5 mM. SDS sample buffer was added at 1 $\times$ and boiled for 10 min. Samples were run on a 15% SDS-PAGE and detected with Coomassie Brilliant Blue R-250.

Western blotting analysis

Sup35NM proteins were analyzed by 15% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). The membranes

were blocked in 5% skim milk for 1 h in PBS buffer with 0.1% Tween 20 (PBST). Primary antibody (1:2500) was added and the membrane was incubated for 1 h, then washed with PBST. Secondary anti-rabbit antibody (1:5000) conjugated to horseradish peroxidase was then added, and the membrane was incubated for 45 min. The proteins were visualized with enhanced chemiluminescence reagent. The development of the signal was recorded on photographic film.

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