

Hyper-efficient PrP^{Sc} Amplification of Mouse-adapted BSE and Scrapie Strain by Protein Misfolding Cyclic Amplification Technique

Aiko Fujihara, Ryuichiro Atarashi, Takayuki Fuse, Kaori Ubagai, Takehiro Nakagaki,

Naohiro Yamaguchi, Daisuke Ishibashi, Shigeru Katamine, and Noriyuki Nishida

The Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.

Corresponding author: Ryuichiro Atarashi, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

Fax: +81-95-819-7060, Tel: +81-95-819-7060, E-mail: atarashi@nagasaki-u.ac.jp

Running title: Hyper-efficient mouse PrP^{Sc} amplification

Abbreviations

PrP^{Sc}, abnormal forms of prion protein; PrP^C, normal cellular form of PrP; TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; mBSE, mouse-adapted BSE; PMCA, protein misfolding cyclic amplification; BH, brain homogenate; NBH, normal brain homogenate; PK, proteinase K; rMoPrP, recombinant mouse PrP.

Summary

The abnormal forms of prion protein (PrP^{Sc}) accumulate via structural conversion of normal PrP (PrP^C) in the progression of transmissible spongiform encephalopathy (TSE). Under cell-free conditions, the process can be efficiently replicated by *in vitro* PrP^{Sc} amplification methods, including the cyclical sonicated amplification (PMCA) technique. These methods enable ultrasensitive detection of PrP^{Sc}, however, there are still difficulties in utilizing them for practical use. For instance, a number of rounds of PMCA have so far been necessary to reach maximal sensitivity, not only taking several weeks, but also resulting in an increase in the risk of contamination. In the present study, we sought to further promote the rate of PrP^{Sc} amplification in the PMCA technique using mouse TSE models infected with either mouse-adapted bovine spongiform encephalopathy (mBSE) or mouse-adapted scrapie, Chandler strain. Here we demonstrate that appropriate regulation of the sonication dramatically accelerates the PrP^{Sc} amplification of both strains. In fact, we reached maximum sensitivity, allowing for the ultrasensitive detection of less than 1LD₅₀ of PrP^{Sc} in the diluted brain homogenates after only one or two rounds of reactions, and in addition, detected PrP^{Sc} in the plasma of mBSE-infected mice. We believe that these results will advance the establishment of a fast, ultrasensitive diagnostic test for TSEs.

Introduction

The transmissible spongiform encephalopathies (TSEs), or prion diseases, are a series of fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle. In the late 1990s, contamination by BSE-infected cattle of the human food chain caused variant CJD (vCJD), mainly in the UK [1, 2]. Moreover, it was reported that vCJD could be transmitted by blood transfusion [3], probably because the species barrier between cattle and man was markedly diminished at secondary transmission. Hence, a blood screening test is urgently needed to prevent the spread of vCJD infection. In addition, early diagnosis is required to provide an opportunity for treatment of TSEs.

The key molecular event in the progression of TSEs is the continuous conformational conversion of the normal cellular form of prion protein (PrP^{C}) into the abnormal isoform (PrP^{Sc}). According to the seeding model hypothesis for prion propagation, PrP^{C} converts into PrP^{Sc} only at the end of PrP^{Sc} polymers [4], indicating that the number of polymers regulates the PrP^{Sc} accumulation rate. An increase in the number of PrP^{Sc} polymers is acquired mainly by the breaking of large PrP^{Sc} polymers into smaller units. Although the *in vivo* factor still remains unknown, the use of sonication to mimic the fragmentation process was successfully applied in the development of an *in vitro* PrP^{Sc} amplification technique, designated protein misfolding cyclic amplification (PMCA) [5]. Using this technique, ultrasensitive PrP^{Sc} detection in easily accessible specimens such as blood and urine was first achieved in a hamster model infected with hamster-adapted scrapie, 263K strain [6-8]. The results would suggest that PMCA is one of the most promising approaches for the development of a blood screening test and the early diagnosis of TSEs. However, a number of rounds of

PMCA have so far been necessary to reach maximal sensitivity [9], not only taking several weeks, but also resulting in an increase in the risk of contamination. Furthermore, although mild amplification has also been demonstrated in several other mammalian species such as mice, cervids, and humans, the amplification levels in these species have been much lower than those in hamster [10-13]. More recently, the addition of a synthetic polyanion, polyadenylic acid, was found to enhance PrP^{Sc} amplification in the PMCA, but the fact that spontaneous formation of PrP^{Sc} was observed after several rounds of reactions could make it difficult to detect genuine PrP^{Sc} in specimens [14, 15]. The use of recombinant PrP as the amplification substrate enabled faster and simpler detection than conventional PMCA methods using brain homogenate [16-20], but attempts to use the blood from TSEs-infected animals as a seed for the amplification assay have not yet been successful. Thus, further studies are required to establish these amplification methods as practical diagnostic assays.

The aim of this study was to seek out conditions that promote PrP^{Sc} amplification using the PMCA technique. We chose mouse-adapted BSE (mBSE) and mouse-adapted scrapie, Chandler strain, as animal models for TSEs. Here, we describe a hyper-efficient amplification of PrP^{Sc} in the two strains achieved by modulating the conditions of sonication.

Results and discussion

The effect of EDTA and digitonin on PMCA

Prior to starting PMCA, we first confirmed the presence of PrP^{Sc} in mBSE-brain homogenate (BH) and Chandler-BH by Western blot analysis. PrP^{Sc} accumulation was detected with mouse monoclonal anti-PrP ICSM35 antibody in both mBSE-BH and Chandler-BH, while none was detected in normal BH (Fig. 1A). The

concentrations of PrP^{Sc} in these BHs were estimated by dot-blotting analysis using recombinant mouse PrP as standards (Fig 1B, C). The average PrP^{Sc} concentrations in the BHs of mBSE and Chandler were 1.21 and 1.86 µg/mg total protein, respectively.

When the conventional PMCA is performed on BHs, EDTA has usually been added to the reaction mixture [9]. In addition, imidazole was previously reported to stimulate PrP^{Sc} amplification in the PMCA system using PrP^C purified from normal BH (NBH) as substrates [21]. Because divalent metal ions, especially copper and zinc ions, are known to inhibit conversion to PrP^{Sc} [21] and fibril formation of recombinant PrP [22], EDTA and imidazole are presumed to minimize the inhibitory actions of the metal ions. Accordingly, we conducted PMCA with or without these chemicals to examine the effect on the amplification. As shown in Fig. 2A, 1-10 mM EDTA was necessary for efficient amplification of Chandler-PrP^{Sc}, while 10-100 mM imidazole had little effect. Similar results were obtained for mBSE-PrP^{Sc} (data not shown). A possible explanation for the results is that many impurities in crude BH may interfere with the action of imidazole, which binds weakly to divalent metal ions, but not that of EDTA, a powerful chelating agent.

We next tested the effect of digitonin on the PMCA, because others have shown that proteinase K (PK)-resistant PrP fragments formed in mouse NBH, and that the formation was inhibited by the presence of 0.05% digitonin [11]. We observed that PK-resistant PrP bands in NBH were clearly detected by SAF83 antibody, which has a conformational epitope located within PrP residues 126-164, but hardly detected by ICSM 35, the epitope of which is located at residues 92-101 (Fig. 2B). On the other hand, both antibodies recognized mBSE-PrP^{Sc} amplified by PMCA (Fig. 2B). The main fragment of the PK-resistant PrP in NBH, designated PrPres^(NBH), was around 25 kDa, that is, smaller than the 27 kDa fragment typical of diglycosylated

PrP^{Sc}. Following serial treatment with PK and PNGase F, a single 16 kDa band of non-glycosylated PrPres^(NBH) was detected, while the fragments of non-glycosylated mBSE- and Chandler-PrP^{Sc} were estimated to be 17 and 18 kDa, respectively (Fig. 2C). The results indicate that the PK-cleavage point of PrPres^(NBH) is positioned more toward the C-terminal than that of PrP^{Sc}. Moreover, the PrPres^(NBH) could be decreased by repeating the sonication, particularly in the presence of 0.05% digitonin (Fig. 2B). In contrast, the amplification and the final quantity of PrP^{Sc} were not affected by digitonin (Fig. 2B, C), indicating that PrPres^(NBH) does not interfere with PrP^{Sc} amplification, and is quite distinct from the spontaneous formation of PrP^{Sc} previously reported [14, 15]. We also found that the formation of PrPres^(NBH) was promoted by the presence of EDTA and detergent (unpublished data). Of note, it has been reported that small amounts of detergent-insoluble and PK-resistant PrP aggregates were present in uninfected human brains in the presence of EDTA and detergent [23]. However, the exact mechanism by which these PK-resistant PrP conformers are generated in NBH remains to be determined. Digitonin, on the other hand, does not appear to enhance the amplification of PrP^{Sc}, but it helps to make the results of PMCA clear, especially when an antibody that recognizes the C-terminal part of PrP is used. After reviewing the results shown in Fig. 2, we decided to add 1 mM EDTA and 0.05% digitonin to the reaction mixture in subsequent experiments.

The influence of durations of sonication on the rate of PMCA

To investigate how conditions of sonication influence the PrP^{Sc} amplification rate, we carried out PMCA with various durations of sonication (5, 10, 20, 40, and 60 seconds) per cycle, using the serially diluted mBSE- or Chandler-BH containing any one of 1 ng (10^{-9} g), 10 pg (10^{-11} g), 100 fg (10^{-13} g), or 1 fg (10^{-15} g)

of PrP^{Sc} as seeds for the reactions. Surprisingly, the rate of PrP^{Sc} amplification varied dramatically according to the duration of sonication (Fig. 3A, B), peaking at 10 seconds of sonication for mBSE and 20 seconds for Chandler, every 30 minutes. Under these conditions, all dilutions of mBSE- or Chandler-BH (from 1 ng to 1 fg of PrP^{Sc}) were readily detectable in a single round (96 cycles, 48 hours) of the reaction (Fig. 3A, B). The results were reproduced in three independent experiments (data not shown). To determine the minimum amount of PrP^{Sc} detectable by the PMCA under the optimal conditions, further dilutions of mBSE-BH and Chandler-BH to 1-10 ag (10^{-18} - 10^{-17} g) of PrP^{Sc} contents were tested. When seeded with mBSE-BH, 2 of 4 replicates with 10 ag PrP^{Sc} and 3 of 4 replicates with 1 ag PrP^{Sc} were detected after one 48-hour round reaction (Fig. 3C). With Chandler-BH, however, only 1 of 4 replicates with 10 ag PrP^{Sc} and none of the replicates with 1 ag PrP^{Sc} was detected (Fig. 3C). After a second serial PMCA reaction, an additional 1 of the 2 replicates with 10 ag PrP^{Sc} of mBSE-BH which were negative in the first round became positive, while the other remained negative (Fig. 3C). Moreover, further rounds did not increase the sensitivity of PrP^{Sc} detection (data not shown). None of the negative controls (no seed) produced detectable PrP^{Sc} bands after the second round of reactions (Fig. 3C), or even after the third and fourth rounds (data not shown), indicating that there was no spontaneous formation of PrP^{Sc} in our PMCA reactions. Although the PMCA experiments were performed very carefully to obtain consistent data, some discrepancies existed in the results shown in Fig. 2C (2 out of 4 for 10 ag vs. 3 out of 4 for 1 ag in the first round seeded with mBSE-BH etc), which may have resulted from positional influence on the delivery of vibrational energy to the samples when very low amounts (1-10 ag) of PrP^{Sc} were used as seeds. Nonetheless, these results provide evidence that the one 48-hour round reaction reached nearly the maximum sensitivity. The efficiencies of PrP^{Sc} amplification in this study were greatly

improved compared with previous studies using Chandler strain, which detected PrP^{Sc} in only 10⁻³ to 10⁻⁴-diluted infected BHs after one round of PMCA [10, 11]. Indeed, we were consistently able to detect 1 fg of PrP^{Sc} (6.5×10⁻¹⁰ dilution of Chandler-BHs). Thus, the increased rate of amplification was at least more than 10⁶-fold. We believe that this increased rate of amplification will contribute to reducing the time required for ultrasensitive detection, and also minimize the risk of contamination.

The approximately 10-fold difference in the sensitivity between mBSE and Chandler may be explained by differences in the minimum size of PrP^{Sc} polymers that can work as seeds for PMCA reactions between the two. Indeed, filtration studies have shown that type 1 and type 2 human PrP^{Sc} have different-sized aggregates [24]. Moreover, it is noteworthy that the quantity of PrP^{Sc} per unit of intracerebral LD₅₀ in mBSE-BH was 7.5 fold less than that in Chandler-BH (4 fg vs. 30 fg PrP^{Sc}), according to our end-point dilution bioassays. These findings may reflect the differences in the size distribution of PrP^{Sc} between the two strains.

The fragmentation of PrP^{Sc} polymers by sonication is generally considered to lead to an increase in the number of PrP^{Sc} polymers, resulting in enhancement of the amplification [5]. However, at the same time, sonication can partially disrupt the PrP^{Sc} aggregate, so that the amplification rate is suppressed in proportion to the disruption. In keeping with this assumption, the infectious titer of sonicated Chandler-BH has been reported to be significantly decreased [25]. Additionally, studies using flow field-fractionation revealed that infectivity and converting activity of PrP^{Sc} purified from 263K-infected hamster brains peaked strikingly in oligomers consisting of 14-28 PrP molecules, while both activities were substantially absent in oligomers of less than 5 PrP molecules [26]. Therefore, hyper-efficient amplification of PrP^{Sc} appears to be achieved by an

appropriate balance between the two opposite effects of sonication on the amplification of PrP^{Sc}.

Ultrasensitive detection of PrP^{Sc} in plasma from mBSE-infected mice

Because plasma is one of the most accessible of specimens, and presumably contains only a very small amount of PrP^{Sc}, we collected plasma samples from four mBSE-infected mice showing clinical signs of TSEs and four uninfected control animals, and performed PMCA to compare seeding activity. In the control reactions, No PrP^{Sc} was seen throughout the first and second rounds (Fig. 4A, B, lanes 5 to 8). In contrast, after only one round of reactions seeded with mBSE plasma, 2 of 4 samples generated clear PrP^{Sc} bands (Fig. 3A, lanes 1 and 2) and a further sample exhibited less distinct bands (Fig. 4A, lane 3). After the second-round reactions, three samples produced strong PrP^{Sc} bands (Fig. 4B, lanes 1 to 3), but the remaining sample still lacked PrP^{Sc} (Fig. 4B, lane 4), and further rounds did not improve the sensitivity (data not shown). The exact reason for the existence of the one negative sample seeded with mBSE-plasma is uncertain, but a possible explanation is that there may be variation of the amount of PrP^{Sc} in plasma among different animals. Furthermore, because we observed that diluted BH frequently lost its seeding activity following freezing and thawing, especially when it contained very low concentrations of PrP^{Sc} (< 1 fg/μl), the freeze-thawing of the plasma may have affected the activity. Nevertheless, these results indicate that the PMCA under optimal sonication conditions is capable of detecting PrP^{Sc} in plasma from mBSE-infected mice within a single round or two rounds of reactions at the most.

Collectively, our findings suggest that ultrasensitive detection of PrP^{Sc} is achievable by one-round PMCA, thereby greatly promoting the opportunities for the development of practical assays for TSEs

including CJD and BSE.

Materials and methods

Substrate preparation for PMCA

Normal brain tissues were isolated from healthy ddY mice (8 weeks old, male), immediately frozen and stored at -80°C . The frozen tissues were homogenized at 10% (w/v) in PMCA buffer (150 mM NaCl, 50 mM HEPES; pH 7.0, 1% Triton X-100, and EDTA-free protease inhibitor mixture [Roche, Mannheim, Germany]) using a Multi-bead-shocker (Yasui Kikai, Osaka, Japan). After centrifugation at $2000 \times g$ for 2 min, the supernatants were collected as normal brain homogenate (NBH) and frozen at -80°C until use. The total protein concentrations in NBH were determined by the BCA protein assay (Pierce, Rockford, IL, USA).

Prion strains

The origin of mBSE was as described previously [27]. The mBSE and Chandler were serially passaged into ddY mice by intracerebral inoculation. The infectious titers were estimated by endpoint titration studies to be $10^{8.5}$ and $10^{7.8}$ LD₅₀ units/g of the brain tissues infected with mBSE and Chandler, respectively. The brains of terminal-stage mice were collected and frozen at -80°C until use. All animal experiments were performed in accordance with the guidelines for animal experimentation of Nagasaki University.

Seed preparation for PMCA

Brain homogenates (BH) derived from mice infected with either mBSE or Chandler strain were prepared as

described above. Dilutions of the seed-BHs were carried out in PMCA buffer immediately prior to PMCA reactions. For plasma collection, blood was collected from the hearts of normal or mBSE-infected mice using a syringe containing EDTA. Blood samples were centrifuged at $2000 \times g$ for 10 min, and the plasma fraction was recovered and stored frozen at -80°C .

Dot blots

BHs and recombinant mouse PrP (23-231) were plotted on nitrocellulose membranes under mild vacuum-assisted conditions using a bio-blot (Bio-rad, Hercules, CA, USA). The membranes were treated with 3 M guanidium thiocyanate for 10 min to denature the proteins. After washing with TBS buffer (10 mM Tris-HCl pH 7.8, 100 mM NaCl) and blocking with 5% skim milk in TBS-T (TBS, 0.1% Tween 20) for 60 min, the membranes were probed with SAF61 anti-PrP monoclonal antibody (SPI bio, Montigny le Bretonneux, France), and the immunoreactive dots were visualized using ECL-plus reagents (GE healthcare, Piscataway, NJ, USA) . The dot intensities were measured for the unit area on the membranes using LAS-3000 mini (Fujifilm, Tokyo, Japan).

Protein misfolding cyclic amplification (PMCA)

To avoid contamination, preparation of non-infectious materials was conducted inside a biological safety cabinet in a prion-free laboratory, and aerosol-resistant tips were used. Substrates (NBH; 7mg/ml) and seeds were prepared in 0.2 ml PCR tube strips as 80- μl solutions containing 1 mM EDTA and 0.05% digitonin, except in the experiments shown in Figure 1 where EDTA and digitonin were omitted as a control. The

diluted mBSE- or Chandler-BH and plasma were used as seeds for the PMCA reactions. To circumvent the influence of sample position on the delivery of vibrational energy to the samples, up to 3 PCR tube strips (24 samples) were placed at the same time in a floating 96-well rack in a sonicator cup horn (Model 3000 with deep-well type microplate horn [Misonix, Farmingdale, NY, USA]) and immersed in 600 ml water in the sonicator bath. The cup horn was kept in an incubator set at 40 °C during the entire PMCA reaction. Sonication was intermittently performed every 30 minutes at 60% power. The durations of sonication are described in the Figure legends.

Proteinase K digestion, SDS-PAGE and Western blotting

After the PMCA reactions, all samples were digested with 20 µg/ml of PK at 37°C for 1 hour. In some experiments, PNGase F (New England Biolabs, Ipswich, MA, USA) treatment was performed after PK digestion. A fourth volume of 5×SDS sample buffer (20% SDS, 10% β-mercaptoethanol, 40% glycerol, 0.1% bromophenol blue and 250 mM Tris-HCl; pH 6.8) was added. Samples (final volume, 32 µl) were then boiled for 5 minutes, loaded onto 1.5-mm 12 or 15% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were probed with ICSM35 (D-Gen, London, UK) or SAF83 (SPI bio, Montigny le Bretonneux, France) or D13 (kindly provided by Dr. B. Caughey, Hamilton, MT, USA) anti-PrP monoclonal antibodies, and visualized using Attophos AP Fluorescent Substrate system (Promega, Madison, WI, USA), in accordance with the manufacturer's recommendations.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Health Labour Sciences Research Grant from the Ministry of Health and Welfare of Japan, and the President's Discretionary Fund of Nagasaki University, Japan. We thank Hitoki Yamanaka and Kazunori Sano for helpful discussions and critical assessment of the manuscript, and Mari Kudo for technical assistance.

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Figure legends

Fig. 1. Estimation of PrP^{Sc} concentration in mBSE-BH and Chandler-BH by dot blot analysis. (A) Detection of PrP in NBH, mBSE-BH, and Chandler-BH without (-) or with (+) PK treatment using Western blots with monoclonal anti-PrP antibody ICSM35. Each lane contains 50 µg of total protein. (B) The designated amounts of recombinant mouse PrP (rMoPrP) were used as standards for the dot blot analysis. The linear regression between dot intensities (arbitrary units) and rMoPrP is shown (n=3, averages ± SD, $r^2=0.967$). (C) NBH, mBSE-BH, and Chandler-BH without (-) or with (+) proteinase K (PK) treatment (40 µg/ml at 37°C for 1 hour) were analyzed (n=3). All three panels were obtained from the same membrane. The regression line in B

represents the concentrations of PrP^{Sc}.

Fig. 2. The effects of EDTA and digitonin on PMCA reactions. (A) The effect of the indicated concentrations of EDTA and imidazole on the PMCA reactions using diluted Chandler-BH containing 1 ng of PrP^{Sc} as seeds. Sonication was performed over a period of 24 hours with 40-second pulses every 30 minutes at 60% power. Samples were digested with PK and probed with ICSM35. (B) The effect of 0.05% digitonin on the PMCA reactions and the formation of PK-resistant PrP in NBH (PrPres^(NBH)). No seed: reaction mixtures containing only NBH were incubated for 24 hours, without (–) or with (+) periodic sonication. mBSE: PMCA with (+) or without (–) digitonin was carried out using diluted mBSE-BH containing 1 ng of PrP^{Sc} as seeds. Sonication was performed as in B. PK-treated samples were analyzed by Western blotting with ICSM35 (epitope located at mouse PrP amino acids [aa] 92–101) or SAF83 (conformational epitope located within aa 126–164). (C) The size differences between PrPres^(NBH) and mBSE- and Chandler-PrP^{Sc} amplified by PMCA with (+) or without (–) digitonin, after consecutive treatments with PK and PNGase F. Samples were probed with SAF83. Molecular mass markers are indicated in kilodaltons on the left.

Fig. 3. Influence of the duration of sonication on the rate of PrP^{Sc} amplification. PMCA was performed at various durations of sonication (5, 10, 20, 40, and 60 seconds) every 30 minutes at 60% power for 48 hours using serially diluted mBSE-BH (A) or Chandler-BH (B) containing the designated amount of PrP^{Sc} as seeds. For reference, 1ng PrP^{Sc} of mBSE and Chandler correspond to 4.7×10^{-4} and 6.5×10^{-4} dilution of infected BHs, respectively. F: frozen control containing 1 ng of PrP^{Sc}. (C) PMCA was performed with 10 second-pulse of

sonication for mBSE and 20 second-pulse for Chandler every 30 minutes for 48 hours. Round 1: first-round of PMCA using serially diluted mBSE-BH or Chandler-BH containing 1 or 10 ag of PrP^{Sc} as seeds. No seed: the same volume of PMCA buffer was added to the reaction mixture as a negative control. All reactions were done in quadruplicate. Round 2: ten percent of each first round reaction volume (8 µl) was used to seed a second round of PMCA. All samples were digested with PK and analyzed by Western blotting with ICSM35.

Fig. 4. Amplification of PrP^{Sc} in plasma of mBSE-infected mice by PMCA. (A) Aliquots (4 µl) of plasma taken from mice in the clinical phase of mBSE (n=4) or normal mice (n=4) were used to seed PMCA reactions. To avoid cross-reaction to mouse immunoglobulins in the plasma, The PrP Fab D13 (epitope aa 96–104) was used to detect PK-digested samples. (B) Second-round reactions were seeded with 10% (8 µl) of each first-round reaction volume and analyzed as in A. rMoPrP: 50 ng of rMoPrP without PK treatment.

Figure 1

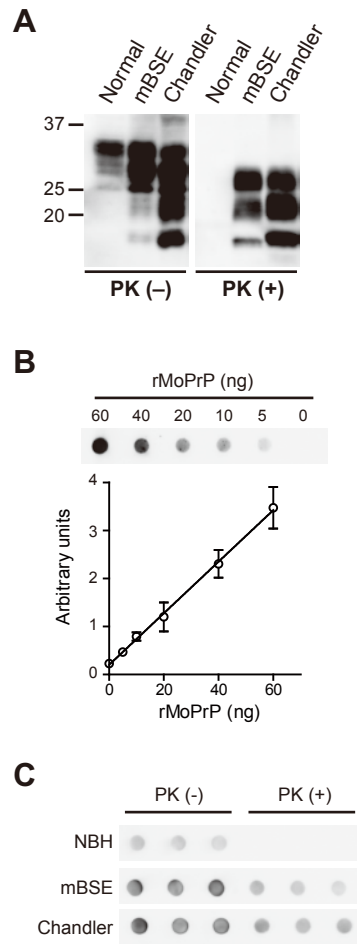


Figure 2

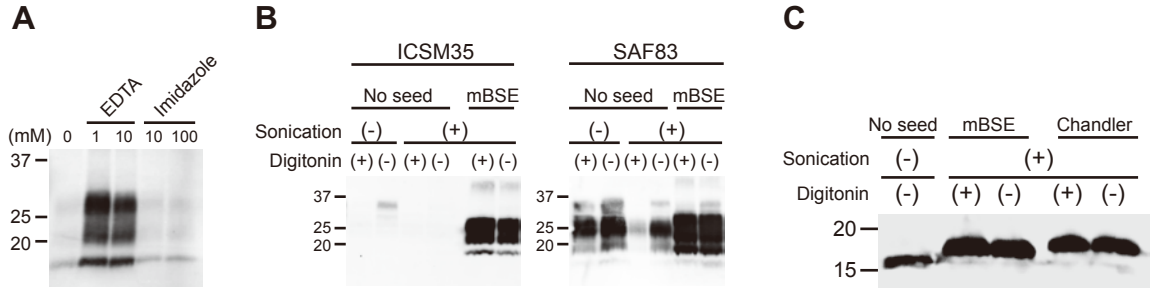


Figure 3

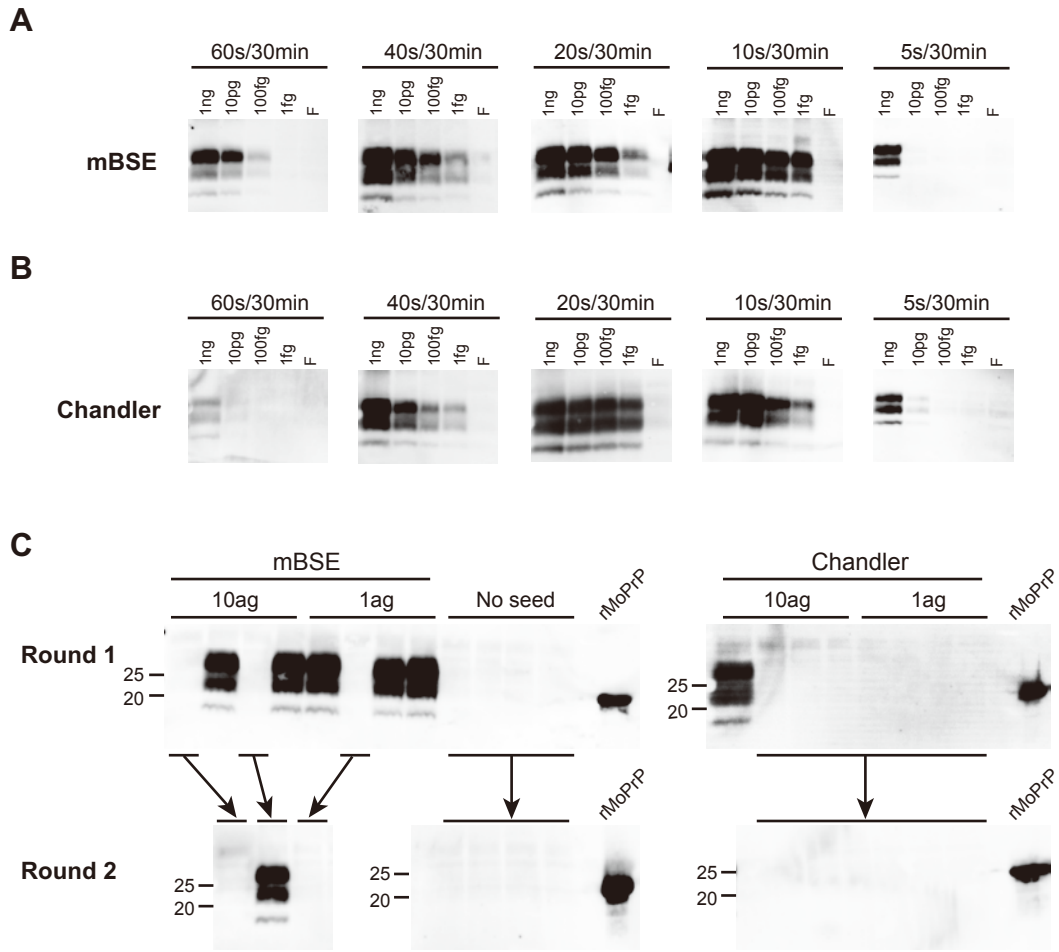


Figure 4

