High-Level Production of High-Purity Human and Murine Recombinant Prion Proteins Functionally Compatible to In Vitro Seeding Assay^S

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Recombinant (rec) prion protein (PrP) is an extremely useful resource for studying protein misfolding and subsequent protein aggregation events. Here, we report mass production of high-purity rec-polypeptide encoding the C-terminal globular domain of PrP; (90-230) for human and (89-231) for murine PrP. These proteins were expressed as His-tagged fusion proteins in *E. coli* cultured by a high cell-density aerobic fermentation method. RecPrPs recovered from inclusion bodies were slowly refolded under reducing conditions. Purification was performed by a sequence of metal-affinity, cation-exchange, and reverse-phase chromatography. The current procedure yielded several dozens of milligrams of recPrP per liter with >95% purity. The purified recPrPs predominantly adopted an α -helix-rich conformation and were functionally sufficient as substrates to measure the seeding activity of human and animal prions. Establishment of a procedure for high-level production of high-purity recPrP supports the advancement of in vitro investigations of PrP including diagnosis for prion diseases.

Keywords: Expression, high cell-density culture, recombinant prion protein, purification, seeding activity

Introduction

PrP^C and PrP^{Sc} are two different conformational isoforms of PrP [1]. PrP^C is a normal cellular protein that is abundantly expressed in neurons, while PrP^{Sc} is an abnormal pathogenic protein that causes progressive neurodegenerative disorders, such as scrapie in sheep,

bovine spongiform encephalopathy in cattle, and CJD in humans [1]. By autocatalytic conformational conversion, the α -helix-rich PrP^C molecule undergoes misfolding and is transformed into the β -sheet-rich PrP^{Sc} molecule [2]. In this biochemical process, PrP^{Sc} functions as a template for PrP^C, resulting in nascent PrP^{Sc} generation [3]. Because misfolded PrP^{Sc} is hydrophobic, it can aggregate in a variety of forms,

such as small amyloidogenic oligomers, unstructured oligomers, amyloids, filaments, protofibrils, and fibrils [4]. These PrP^{Sc} aggregates constitute prion particles that exhibit infectivity [5].

Because the molecular events involved in autocatalytic PrP^{Sc} generation and aggregation of PrP^{Sc} molecules confer propagation of prion infectivity, production of prion infectivity *de novo* is the ultimate proof of the protein-only hypothesis in prion biology [6]. Experiments to evaluate the infectivity of misfolded protein aggregates have been performed with defined materials, such as recPrP expressed in bacteria [6, 7]. Furthermore, recPrP has been used as a source of antigens to raise anti-PrP antibodies [8–14]. Highly purified recPrP itself is essential to resolve the three-dimensional structure of PrP in various conformations [15–21]. Demand for recPrP has increased as it has become the major material for *in vitro* PrP aggregation assays, such as the PrP amyloid formation assay (PAFA) or real-time quaking assay (RT-QuIC), which are used to detect the “seeding activity” of prions [22–24]. These assays are powerful tools for prion diagnosis as they measure acceleration of PrP amyloid formation initiated by misfolded PrP conformers as seeds.

A number of studies have described expression and purification of recPrP in *E. coli*, *Pichia*, and cultured insect cells [8, 25–35]. Although bacterially expressed recPrP lacks posttranslational modifications, the *E. coli* system is most widely used to produce recPrP. Because PrP^C is a membrane protein tethered to the plasma membrane via a glycosylphosphatidylinositol anchor and associated with lipid- and/or cholesterol-rich domains of the plasma membrane [36, 37], production of recPrP as a soluble recombinant protein is difficult. Thus, recPrP is usually expressed as an insoluble protein in inclusion bodies within the bacterial cells and then solubilized for purification [8, 26, 27, 34, 35, 38]. However, preparation of purified recPrP is still a difficult task because of the intrinsic characteristics of PrP. This protein is notorious for its non-specific interactions with proteins and other biological macromolecules as well as the surfaces of non-biological materials, such as steel and plastic [39–42]. Affinity tagging of recPrP makes purification easier. Usually, recPrP is expressed as a protein with a fusion tag and is purified by single-step affinity chromatography [8, 25–27, 33, 34, 43, 44], although recPrP can be expressed and purified without a tag via multiple chromatography steps [35]. In addition, PrP tends to be partially precipitated during refolding performed after purification [35]. Although

slow refolding of diluted recPrP in acidic pH may minimize the appearance of insoluble aggregates [45], the loss of a considerable amount of recPrP results in a low concentration of refolded protein [34, 35, 46–48]. Thus, an additional step to concentrate recPrP is frequently needed.

Nonetheless, some studies have reported the large-scale preparation of recPrP [34, 35], while others have described the production of highly pure recPrP [27]. However, it is still difficult to obtain a large quantity of highly pure recPrP using existing expression and purification procedures. Problems associated with the aforementioned intrinsic properties of recPrP, loss of expressed protein during purification, refolding, and concentration processes, and the limitations of low density cell cultures, leading to a small mass of starting material, have hindered the production of concentrated, high-purity recPrP.

To address the problems associated with production of a large quantity of highly pure recPrP, we report the mass-production of high-purity His-tagged human (h) and mouse (m) recPrP spanning the C-terminal core region (amino acids 90–230 for human and 89–231 for mouse; Fig. S1) based on the combined approach of high cell-density aerobic fermentation and a series of chromatography steps for purification. Furthermore, we provide evidence that recPrP prepared by the present methodology is suitable as a substrate to measure the seeding activity of prions.

Materials and Methods

Cloning of PrP Genes in Bacterial Expression Vectors

For expression of His-tagged [(His)₆] hPrP(90–230) and mPrP(89–231), DNA fragments encoding hPrP(90–230) and mPrP(89–231) were cloned into the bacterial expression vector, pET100/D-TOPO (Invitrogen, USA), as described previously [23]. Human and mouse PrP DNA fragments were obtained by PCR, using the complete DNA sequences for the PrP genes cloned in the pSP72 cloning vector as templates. PCR primers were as follows: for hPrP(90–230) gene cloning, forward primer - 5′ CTA CGA TCC TCT CTG GTA ATA 3′; reverse primer - 5′ TCA TCC CAC TAT CAG GAA GAT GAG 3′; for mPrP(89–231) gene cloning, forward primer - 5′ CAC CGG CCA AGG GGG TAC CCA 3′; reverse primer - 5′ TCA GCT GGA TCT TCT CCC GTC GTA 3′. PCR was performed under the following conditions: 1 cycle of 95°C for 10 min, 35 cycles of 95°C for 30 sec/58°C for 30 sec/72°C for 1 min, and 1 cycle of 72°C for 7 min. The resultant recombinant plasmids were used to transform *E. coli* BL21 Star(DE3) (Invitrogen). This was accomplished according to the manufacturer’s instruction and a modified protocol described previously [23]. Recombinant plasmids harboring PrP DNA fragments were sequenced to

confirm cloning of corresponding gene fragments with no unintended mutations (Cosmo Genetech, Korea).

Expression of (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) Using High Cell-Density Aerobic Fermentation

The overall scheme for expression and purification is depicted in Fig. S2. *E. coli* BL21 Star(DE3) cells transformed with recombinant plasmids for (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) were cultured in 25 ml LB medium supplemented with 50 µg/ml ampicillin (Sigma-Aldrich, USA) at 37°C as seed cultures. When the OD₆₀₀ of the cultured cell broth was 0.7, the seed culture was used to inoculate 1 L of fermentation medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, K₂HPO₄ 5 g/l) in an aerated 2.5 L fermentor (Sartorius Stedim Biotech, France). After 15–16 h of growth at 25°C, the temperature was raised to 30°C to induce expression of (His)₆-hPrP(90-230) and to 37°C to induce expression of (His)₆-mPrP(89-231). Induction was performed by addition of IPTG (Invitrogen) to a final concentration of 1 mM when the OD₆₀₀ was over 10. During aerobic fermentation (hereafter, fermentation), glucose and NH₄OH were used as nutrients and pO₂ was kept to 30% to maintain the pH at 6.9.

Cell Lysis and Washing of Inclusion Bodies Containing (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231)

Cells were harvested by centrifugation at 12,000 ×g at 4°C for 30 min. Cell pellets were resuspended in 20 ml of resuspension buffer (10% sucrose, 0.1 M Tris, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.2 M NaCl, pH 7.9) per 1 g of cell weight. Resuspended cells were disrupted using a homogenizer (SPX FLOW, USA) and centrifuged at 12,000 ×g at 4°C for 30 min. The supernatant was discarded and the pellet, comprising inclusion bodies containing (His)₆-hPrP(90-230) or (His)₆-mPrP(89-231), was collected. The pellet was washed with 30 ml of inclusion body washing buffer (20 mM Tris, 1 mM EDTA, 0.02% lysozyme, 1% Triton X-100, 0.5 M urea) per 1 g of pellet for 2 h. The inclusion body wash was repeated once again and the final wash was completed with deionized water in the same manner.

Direct Refolding of Recombinant (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231)

Washed inclusion bodies were dissolved in solubilization buffer (8 M urea, 10 mM glycine, pH 10.6). The inclusion body solution was diluted with refolding buffer (0.6 M urea, 10 mM glycine, pH 10.6) to a final protein concentration of 0.5 mg/ml and incubated at 4°C for 16 h with 0.1 mM β-mercaptoethanol. Refolded recPrP solution was adjusted to 20 mM Tris, 0.5 M NaCl, 5% glycerol.

IMAC

IMAC was performed with refolded recPrPs using a HisPrep FF 16/10 column (GE Healthcare Bio-Sciences, USA). The protein solution was loaded at a flow rate of 3 ml/min on a 20 ml HisPrep FF 16/10 column equilibrated with five CVs of binding buffer (50 mM Tris, 0.5 M NaCl, 5% glycerol, pH 8.0) at a flow rate of

5 ml/min. The column was washed with five CVs of washing buffer (20 mM Tris, 0.5 M NaCl, 5% glycerol, 100 mM imidazole, pH 8.0) at a flow rate of 5 ml/min. Bound proteins were eluted with two CVs of elution buffer (20 mM Tris, 0.5 M NaCl, 5% glycerol, 1 M imidazole, pH 8.0) at a flow rate of 5 ml/min. The eluent was collected in 5 ml aliquots in fraction tubes.

Cation Exchange Chromatography

IMAC eluate fractions containing recombinant (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) were pooled and subjected to cation exchange chromatography using the AKTA Avant system (GE Healthcare Bio-Sciences) equipped with a 20 ml Sp Sepharose fast flow resin (GE Healthcare Bio-Sciences) in an XK 16 column (GE Healthcare Bio-Sciences). The pooled samples were desalted with equilibrium buffer (7 M urea, 0.25 M acetic acid, pH 2.5) using Stirred Ultrafiltration Cells containing a 3 kDa ultrafiltration membrane (Millipore, USA). The column was equilibrated with 10 CVs of equilibrium buffer at a flow rate of 3 ml/min, loaded with the pooled sample at a flow rate of 1 ml/min, and washed with 10 CVs of equilibrium buffer at a flow rate of 3 ml/min. Then, bound proteins were eluted with 20 CVs of equilibrium and elution buffers (7 M urea, 0.25 M acetic acid, 1 M NaCl, pH 2.5) by application of a linear gradient (0–1 M NaCl) at a flow rate of 3 ml/min. During this process, the solution was monitored at 280 nm and each peak was collected in fraction tubes.

Prep-HPLC

Cation exchange chromatography fractions were further purified by reverse phase chromatography. For this purification step, Prep-HPLC was carried out on an Agilent 1200 system (Agilent Technologies, USA) equipped with a C8 prep HT column (21.2 mm × 150 mm, particle size 5 µm, Agilent Technologies). Solvent A comprised 0.1% TFA in deionized water and solvent B comprised 0.09% TFA in ACN. The column was equilibrated with 10 CVs of solvent A at a flow rate of 5 ml/min and was loaded with the pooled sample at a flow rate of 1 ml/min. After washing with 10 CVs of solvent A at a flow rate of 5 ml/min, bound proteins were eluted with 10 CVs of solvents A and B at a flow rate of 5 ml/min by application of a linear gradient (0–100% solution B). During this process, the solution was monitored at 214 nm and each peak was collected.

CD Spectrometry

The secondary structures of refolded (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) were determined by CD spectrometry. RecPrP was diluted to 1 mg/ml in 20 mM sodium acetate (pH 5.5). CD spectra of the samples in cells of 1 mm path length were measured using a Chirascan circular dichroism spectrometer (Applied Photophysics, UK). CD data were collected from 190 to 260 nm with a 1 nm bandwidth at 25°C and are expressed as the mean residue ellipticity from three scans collected on a sample. CD results of (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) were confirmed by at least three independent readings.

SDS-PAGE and Western Blotting

Protein samples collected during expression and purification steps were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. These samples were also analyzed by western blotting using monoclonal anti-(His)₆ tag antibody (1:1,000 dilution, Abcam, UK), and anti-PrP antibodies 6D11 (1:30,000 dilution, BioLegend, USA) and 3F4 (1:10,000 dilution, BioLegend). Goat anti-mouse IgG (H+L) antibody conjugated with horseradish peroxidase (1:5,000 dilution, Thermo Scientific Korea, Korea) was used as the secondary antibody. Using the G:Box Chemi XR5 system and GeneTools software (Syngene, UK), chemiluminescent detection of recPrP was performed with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences).

Analytical HPLC to Estimate Purity

To estimate the purity of the recPrPs, samples were analyzed using a Waters HPLC system (USA) equipped with a SKYPAK C8 analytical column (250 nm × 4.6 mm, particle size 5 µm, SK Chemicals, Korea). Buffer A comprised 0.1% TFA in deionized water and buffer B comprised 0.09% TFA in ACN. The analysis was performed under a linear gradient of 0% to 80% buffer B to buffer A at a flow rate of 1 ml/min for 30 min. Twenty µl of the samples were injected and monitored at 280 nm by UV detector.

Measurement of Prion Seeding Activity

PAFA was performed in the presence of misfolded PrP molecules from various human (IRB No: 2016-01-05-R-A) and mouse (IACUC No: 2017-0017A) sources to measure seeding activity. Briefly, the PAFA reaction included 0.2 µM recPrP and 2 µl of seeds in 200 µl reaction buffer (PBS, pH 7.0, 0.2 M guanidine hydrochloride and 10 µM ThT) [23]. Seeds were initially prepared by a thousand-fold dilution of prion-infected and -uninfected normal biological matter in PBS and, then, a hundred-fold further diluted in PAFA reactions in a homologous manner. Homogenates of the brains of sporadic CJD patients and normal individuals deposited in the Korea Centers for Disease Control and Prevention (Korea) were used as seeds for (His)₆-hPrP(90-230) aggregation. Similarly, homogenates of terminally ill prion-infected and normal mice brains, as well as prion-infected (ScN2a) and uninfected (N2a) neuroblastoma cells, were used as seeds for (His)₆-mPrP(89-231) aggregation. PAFA was performed in 96-Well Black UniPlate microplates (GE Healthcare) by incubating the plates at 37°C for 48 h with shaking at 300 rpm. Formation of PrP aggregates was monitored every 5 min by reading the fluorescence of the ThT bound to amyloids using fluorescence microplate readers equipped with excitation (444 nm) and emission (485 nm) filters. With regard to biosafety measures, PAFAs for human samples were performed using a FLUOstar Omega microplate reader (BMG Labtech GmbH, Germany) in the BL-3 laboratory of the Korea Centers for Disease Control and Prevention (Korea). PAFAs for mouse samples were performed using an Infinite M200Pro Multimode Reader (Tecan, Switzerland) in a BL-2 laboratory at Hanyang University (Korea).

Results

Production of Recombinant Plasmids for Bacterial Expression of (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231)

The 423 bp- and 429 bp-long PrP gene fragments encoding the C-terminal globular domain of human and mouse PrP^C, respectively, were PCR-amplified (Fig. S3A) and cloned in pET100/D-TOPO (Fig. S3B). Successful cloning was evidenced by the fact that the sum of the PCR product size (~430 bp) and the sequence span between NheI and SacI sites (~130 bp) in the vector was comparable to the size of the insert DNA fragments when the recombinant plasmids were digested with the corresponding restriction enzymes. Separately, DNA sequencing of recombinant plasmids confirmed the cloning of gene fragments for the C-terminal globular domain of human and mouse PrP^C.

Expression of (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) during Fermentation

Fermentation to express (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) was performed for 23 and 25 h, respectively. Protein expression was induced when the OD₆₀₀ reached 15.6 for (His)₆-hPrP(90-230) and 13.3 for (His)₆-mPrP(89-231) by adding IPTG to a final concentration of 1 mM. Cell culture was continued for an additional 6 h for (His)₆-hPrP(90-230) and 4 h for (His)₆-mPrP(89-231). The final OD₆₀₀ reached 21.3 for (His)₆-hPrP(90-230) and 24.0 for (His)₆-mPrP(89-231) (Fig. 1A and 1B). The wet cell weights obtained were 28.6 for (His)₆-hPrP(90-230) and 46.9 for (His)₆-mPrP(89-231). Sample aliquots were collected at several time points after raising the temperature to 30°C or 37°C for (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231), respectively, to determine expression level during the time course of cell culture. SDS-PAGE analysis showed that expression of (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) was induced after addition of IPTG (Fig. 1C and 1D). The molecular weight of both (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) was ~19 kDa.

Expressed Recombinant (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) in Inclusion Bodies

Inclusion bodies were prepared by separating insoluble protein aggregates from disrupted cells. These aggregates were washed with washing buffer and deionized water. SDS-PAGE analysis showed that (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) were present in the inclusion bodies, although a number of non-PrP protein species were included at a low level (Fig. 2A and 2B, Lanes 1–5). The purity of

Fig. 1. Cell culture and SDS-PAGE analysis of recPrP expression.

Fermentation and growth curves of (His)₆-hPrP(90-230) (A), and (His)₆-mPrP(89-231) (B). The cells for (His)₆-hPrP(90-230) were cultured for 15 h at 25°C and expression was induced with 1 mM IPTG for 6 h at 30°C. The cells for (His)₆-mPrP(89-231) were cultured for 16 h at 25°C and expression was induced with 1 mM IPTG for 4 h at 30°C. SDS-PAGE analysis of expressed (His)₆-hPrP(90-230) (C) and (His)₆-mPrP(89-231) (D) according to indicated culture time. Arrows indicate the addition of IPTG.

recPrPs in the inclusion bodies increased progressively during repeated washing.

Purification of (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) by Sequential Chromatography

RecPrP was first purified by nickel affinity chromatography.

Proteins bound to the resin were washed with washing buffer containing 100 mM imidazole. The fusion protein was eluted with elution buffer containing 1 M imidazole and the fractions were collected. Analysis of pooled fractions by SDS-PAGE demonstrated that highly pure recPrPs were enriched by IMAC (Fig. 2A and 2B, Lane 6).

Fig. 2. SDS-PAGE analysis of recPrPs during inclusion body washing and IMAC.

(A) (His)₆-hPrP(90-230). (B) (His)₆-mPrP(89-231). Lane 1: Total cell lysate of induced recPrPs, Lane 2: Pellet, Lane 3: The first wash of inclusion body, Lane 4: The second wash of inclusion body, Lane 5: Solubilized inclusion body, Lane 6: Pooled fraction containing recPrPs from IMAC.

Fig. 3. Purification of recPrPs using cation-exchange chromatography.

Cation-exchange chromatograms of (His)₆-hPrP(90-230) (A) and (His)₆-mPrP(89-231) (C) with a HiTrap Sp column. Arrows indicate recPrPs (1), percentage of buffer B (%) (2), and conductivity (3). HPLC chromatogram of (His)₆-hPrP(90-230) (B) and (His)₆-mPrP(89-231) (D).

To improve the purity of recPrPs, pooled IMAC elution fractions were subjected to cation-exchange chromatography. During elution of loaded HiTrap SP columns with a linear gradient, ranging from 0 to 1 M NaCl, the major peak of recPrPs was detected at around 0.8–1 M NaCl for (His)₆-hPrP(90-230) and 0.75–0.9 M NaCl for (His)₆-mPrP(89-231) (Fig. 3A and 3C). Based on the HPLC chromatogram obtained using the Protein & Peptide C8 analytical column, the purity of (His)₆-hPrP(90-230) was 91.5% and that of (His)₆-mPrP(89-231) was 91.4% (Fig. 3B and 3D).

As a final chromatography step to increase the purity of recPrPs, the pooled PrP solution prepared from cation exchange chromatography was loaded on a reverse-phase chromatography column. Elution was accomplished with a linear gradient containing 0% to 70% ACN. The Prep-HPLC chromatogram of the C8 prep HT column showed that the main peak of recPrPs was found in the range of 43–45% ACN for (His)₆-hPrP(90-230) and 38–42% ACN for (His)₆-mPrP(89-231) (Fig. 4A and 4C). SDS-PAGE of recPrPs found in the main peak showed pure recPrPs as a single

band in the gel with no other impure protein bands (Fig. 4B and 4D).

RecPrP production from cells fermented to a high density followed by multiple chromatography purification steps yielded 72 and 60 mg per liter for (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231), respectively.

Confirmation of Purified recPrPs by Immunoblotting

To confirm the identity of the purified recPrPs, western blotting using different antibodies was performed. Reactivity of both (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) with anti-(His)₆ antibody confirmed that the recPrPs were expressed as His-tagged fusion proteins (Fig. S4A). Detection of both (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) by anti-PrP 6D11 antibody, but failure to detect (His)₆-mPrP(89-231) using the anti-PrP 3F4 antibody, confirmed that the purified recombinant proteins represented their corresponding PrPs (Fig. S4B and S4C), because the epitope of 3F4 is available for antibody binding in hPrP, but not mPrP [49].

Fig. 4. Preparative HPLC chromatography and SDS-PAGE analysis of purified recPrPs.

Preparative HPLC chromatograms of (His)₆-hPrP(90-230) (A) and (His)₆-mPrP(89-231) (C) with C8 prep HT column. SDS-PAGE analysis of pooled fractions containing (His)₆-hPrP(90-230) (B) and (His)₆-mPrP(89-231) (D).

Secondary Structure of Refolded (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231)

To assess whether refolding performed during purification allowed the recPrPs to retain a conformation similar to that

of native PrP^C, the secondary structures of (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) were analyzed by CD spectrometry. CD spectra of both recPrPs demonstrated a positive maximum at 193 nm and double negative maxima at 208 and 220 nm (Fig. S5). These results indicated that both (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) adopted the typical α -helix-rich conformation.

Purity of Purified (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231)

The purity of the final (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) products was determined by analytical HPLC with a Protein & Peptide C8 analytical column. The HPLC chromatogram showed a single peak with a retention time at ~20 min for both recPrPs (Fig. S6). Calculation of the integrated area under the peak indicated that the purity of (His)₆-hPrP(90-230) was 95.7% while that of (His)₆-mPrP(89-231) was 95.8%.

Utility of Produced recPrPs as Substrates to Measure the Seeding Activity of Prions

To estimate whether recPrPs generated by the method described in the present study are suitable to measure the seeding activity of prions, (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) were tested as substrates in PAFA. In the presence of seeds prepared from a CJD brain, the length of the lag phase for aggregation of misfolded (His)₆-hPrP(90-230) (~17.5 h) was reduced by 4.5–9 h compared to the lag phase obtained in the absence of seeds (~26.5 h) or in the presence of seeds prepared from normal brains (~22 h) (Fig. 5A). Similarly, aggregation of misfolded (His)₆-mPrP(89-231) was also accelerated 8–9 h and 6–9 h by seeds prepared from prion-infected cells and mouse brains, respectively

Fig. 5. Application of purified recPrPs to assays to measure prion seeding activity.

PAFA using (His)₆-hPrP(90-230) was performed with CJD/normal brain seeds (A). PAFA using (His)₆-mPrP(89-231) was performed with ScN2a/N2a (B) and prion-infected/uninfected brain seeds (C).

(Fig. 5B and 5C). Although irrelevant to the measurement of seeding activity, the amplitudes of fluorescence measured in PAFA with or without misfolded seeds varied (Fig. 5). These results demonstrate that both (His)₆-hPrP(90-230) and (His)₆-mPrP(89-231) generated in this study can be reliably used in assays to evaluate the seeding activity of prions.

Discussion

The current study reports the technical establishment of a method to mass-produce highly pure recPrP by using high cell-density fermentation and three sequential chromatographic purification procedures. This study simultaneously achieved two essential goals of recPrP production, a high purity and a large quantity. Supply of highly pure recPrP in large quantity is important for prion detection using the assay platform of *in vitro* PrP aggregation [50]. Thus, development of a strategy to produce high-quality recPrP is advantageous for dependable prion detection tests, which rely on a large quantity of substrate.

E. coli is the most widely used host cell to produce recombinant proteins because of its unparalleled fast growth kinetics [51], easy transformation with exogenous plasmid DNA [52], and low culture costs. High cell-density fermentation can easily be performed and produces a large microbial cell mass [53]. However, during this process, cells encounter disadvantageous conditions such as exhaustion of dissolved oxygen, nutrient depletion, changing pH, elevated osmotic pressure, and byproduct formation. To solve these problems, feeding strategies such as DO-stat and pH-stat have been adopted in fed-batch fermentation [54]. In this study, the established procedure to produce recPrPs employed fed-batch, high cell-density fermentation using pH-stat. Thus, a large cell mass could be obtained and purification was initiated with a copious amount of initial starting material, which resulted in production of recPrP in a large quantity. In fact, comparison of recPrP production from cells fermented to a high density followed by multiple chromatography purification steps to that from conventional liquid culture with a single affinity chromatography purification step indicates that recPrP production based on the former described in this study is more efficient than that by the latter described elsewhere [8, 23]. In the case of (His)₆-mPrP(89-231) production, the yield increased ~30-fold. For an identical volume of cell culture, the final OD₆₀₀ and wet cell weight were greater by more than 20-fold.

Purity is a crucial factor for experiments that use recPrPs.

Although IMAC effectively enriches His-tagged recPrPs, a certain level of non-recPrP material is believed to be included in the eluates. To exclude impurities, two additional purification steps, cation exchange and reverse-phase chromatography, were used in this study. Indeed, analyses of analytical HPLC chromatograms demonstrated that the purity of recPrP purified by cation exchange chromatography was 91.5% for hPrP(90-230) and 91.4% for mPrP(89-231), and that the purity of recPrP purified by reverse-phase chromatography was 95.7% for hPrP(90-230) and 95.8% for mPrP(89-231). These data indicate that sequential application of different chromatography steps successfully increased the purity of recPrP to a high level. Usually, purification of recPrP is achieved by one- or two-step chromatography [8, 25, 30, 34, 35, 43]. RecPrP produced in our study is of high quality and will be a useful resource for studies that require recPrP. In particular, use of recPrP substrates free of other macromolecules is critical for seeding assays. Some macromolecules, such as lipids and nucleic acids, have been shown to assist in PrP conversion as cofactors [7, 55, 56]. Exclusion of such contaminants helps establish precise assays.

One of the unavoidable drawbacks involved in refolding of recPrP prepared using the conventional method is the considerable loss of protein by precipitation. This decreases the amount of recPrP that can be obtained as a final product, despite successful purification. It is common to lose recPrP when the protein is purified first and refolded afterwards. The protocol used in the current study includes repeated denaturation and renaturation; refolding of denatured recPrP in the presence of low concentrations of denaturant and reducing agent, temporal denaturation of refolded recPrP with the high concentration of denaturant in the absence of reducing agent during ion exchange chromatography, and then, final renaturation at the end of purification. The order of processes in this study explains how the massive loss of recPrP could be prevented, improving the yield per cell mass.

Highly pure recPrPs mass-produced in this study properly served as substrates in PAFA to measure seeding activity, the indication of aggregation of which initiation is accelerated by the presence of misfolded PrP isoforms. However, the correlation between recPrPs used in PAFA and the level of fluorescence which represents the degree of aggregation in the current study was not established. Traditionally, the level of misfolded conformer in seeds is determined by measuring the ability to initiate aggregation, which is proportional to the abbreviated lag phase of PrP aggregation, but not by measuring the level of aggregated PrP. Regardless,

whether the quality of substrates in PAFA influences a high-degree generation of PrP aggregates under a certain seeding condition would be interesting to investigate.

In conclusion, we expressed and purified large quantities of highly pure recPrPs using fed-batch fermentation and sequential chromatography steps. The recPrPs generated showed conformational similarity to native PrP^C and supported PrP aggregation, one of the major events that characterizes prion diseases. Establishment of such a procedure can help address the issues associated with the production of large quantities of highly pure recPrP.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

Abbreviations

PrP^C, cellular prion protein; **PrP^{Sc}**, scrapie prion protein; **CJD**, Creutzfeldt-Jakob disease; **LB**, Luria-Bertani; **OD₆₀₀**, optical density at 600 nm; **IPTG**, isopropyl β -D-1-thiogalactopyranoside; **IMAC**, Immobilized metal affinity chromatography; **CVs**, column volumes; **Prep-HPLC**, Preparative high-performance liquid chromatography; **TFA**, trifluoroacetic acid; **ACN**, acetonitrile; **CD**, circular dichroism; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **PBS**, phosphate buffered saline; **ThT**, thioflavine T

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