Published in final edited form as:

Prep Biochem Biotechnol. 2012 ; 42(1): 77–86. doi:10.1080/10826068.2011.564256.

NON-REDUCING ALKALINE SOLUBILIZATION AND RAPID ON-COLUMN REFOLDING OF RECOMBINANT PRION PROTEIN

Daniel J. Walsh¹, Geoffrey P. Noble¹, Justin R. Piro¹, and Surachai Supattapone^{1,2}

¹Departments of Biochemistry, Hanover, New Hampshire, USA

²Departments of Medicine, Dartmouth Medical School, Hanover, New Hampshire, USA

Abstract

Mature prion protein (PrP) is a 208-residue polypeptide that contains a single disulfide bond. We report an alternative method to purify recombinant mouse PrP produced in *Escherichia coli*. Bacterial inclusion bodies were solubilized in a buffer containing 2 M urea at pH 12.5. The solubilized protein was rapidly purified on a nickel affinity column without a chaotrope gradient, followed by ion-exchange chromatography. The yield and purity of PrP produced by this alternative approach was similar to that obtained using a conventional solubilization and on-column refolding protocol. Recombinant PrP produced using the non-reducing purification protocol is properly folded, as determined by circular dichroism, and a competent substrate for amyloid fibril formation, as determined by Thioflavin-T dye binding assays. In summary, this report describes a rapid method for producing properly folded recombinant PrP without reducing agents or a chaotrope gradient.

Keywords

non-reducing; on-column; prion; PrP; purification; recombinant; solubilization

INTRODUCTION

Unorthodox proteinaceous infectious agents called prions cause a variety of fatal human and animal diseases, such as Creutzfeldt–Jakob disease (CJD), chronic wasting disease (CWD), bovine spongiform encephalopathy (BSE), and scrapie, collectively known as the transmissible spongiform encephalopathies (TSEs). The central pathogenic event in these diseases is the conformational change of a normal cellular prion protein PrP^C into a misfolded form termed PrP^{Sc}.^[1] Mature PrP is a 208-amino-acid polypeptide with two asparagine-linked glycans, a C-terminal glycosylphosphatidylinositol (GPI) anchor, and a single intramolecular disulfide bond between cysteine residues 179 and 214.^[2–4] Although PrP^C is ~42% α -helical with minimum β -sheet content, PrP^{Sc} contains 30% α -helix and 43% β -sheet.^[7] Currently, the vast majority of research effort to understand and combat TSEs is focused upon studying PrP. In addition to its role as a substrate for prion formation, PrP^C may also serve as a receptor for A β oligomers in Alzheimer's disease.^[2]

Recombinant PrP (recPrP) expressed in bacteria can be refolded in vitro into a tertiary conformation similar to that of PrP^C, despite lacking the glycan modifications of the native protein.^[7–9] Recombinant PrP is an essential reagent for a variety of diagnostic protocols

that are based on the ability of prions to template conformational change.^[10] The availability of recPrP has also greatly facilitated mechanistic studies of prion protein structure and amyloid fibril formation,^[11–16] and it has recently been reported that infectious prions can be formed *de novo* from recPrP substrate.^[17]

Several different protocols have been developed to solubilize, purify, and refold recPrP from bacterial inclusion bodies.^[10,18–20] Most of these methods use reducing conditions and a high concentration of chaotropic agents to solubilize inclusion bodies containing recPrP. One convenient method employs a decreasing gradient of guanidine to refold recPrP on a Ni-NTA affinity column under non-reducing conditions.^[10] Previously, Patra et al. showed that using a 2 M urea solution at pH 12.5 to solubilize inclusion bodies containing recombinant human growth hormone (recHGH) preserves native-like secondary structure, reduces aggregation, and improves the yield of bioactive recHGH.^[21] Here, we examined whether these solubilization conditions could be combined with an on-column refolding protocol for producing recPrP, a conformationally flexible, aggregation-prone protein with an unstructured N-terminus. We report that non-reducing solubilization with alkaline 2 M urea facilitates rapid column purification (without using a chaotrope gradient) and production of properly folded recPrP.

MATERIALS AND METHODS

Materials

All chemicals and reagents were acquired from Fisher Scientific (Pittsburgh, PA), unless otherwise specified. Aqueous solutions were produced using deionized, distilled water from a Milli-Q filtration system (Millipore, Billerica, MA).

Plasmid Construction and Transformation

Amplified DNA sequences coding for mouse PrP 23-231 were ligated into the pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Mouse PrP was then cut out of the pCR2.1-TOPO vector using XHO1 and NDE1 restriction enzymes (NEB, Ipswich, MA) and ligated into the pET-22b(+) expression vector (EMD Chemicals, Gibbstown, NJ) and sequences were verified. The expression vector was then transformed into *Escherichia coli* Rosetta cells (EMD Chemicals). Cells were mixed with 15% glycerol and stored at -70°C .

Expression and Isolation of Inclusion Bodies

Rosetta cells were inoculated into 1 L Terrific Broth (Sigma-Aldrich, St. Louis, MO) and incubated overnight at 37°C , shaking at 200 rpm in a series 25 incubator shaker (New Brunswick Scientific Co., Edison, NJ). Expression was induced using the Novagen Overnight Express Autoinduction System 1 (EMD Chemicals). After incubation, cell pellets were collected by centrifugation and resuspended in 40 mL of $1 \times$ cell lysis buffer, BugBuster Protein Extraction Reagent (EMD Chemicals) with Roche Complete, EDTA-free, Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN), and Lysoase Bioprocessing Reagent (EMD Chemicals). The suspension was incubated on ice for 30 min with intermittent direct probe sonication (30-s bursts every 5 min) to lyse cells and solubilize debris. After centrifugation, the pellet was washed twice with 40 mL $0.1 \times$ cell lysis buffer to remove any residual lysate. At this point, the inclusion-body pellet can either be solubilized or frozen at -20°C for later use.

Assay for Optimal Solubilization Conditions

An efficient solubilizing buffer was identified by screening the ability of several solutions to extract monomeric protein from recPrP inclusion bodies on a small scale. Briefly, inclusion

body pellets equivalent to 25 mL of bacterial culture were resuspended in 1 mL of buffer and then incubated at room temperature for 30 min before measuring the turbidity of the solution at 562 nm. Next, following a clarifying spin, the absorbance of the solution was measured at 280 nm to estimate the amount of soluble protein present. This method was applied to solutions buffered with 10 mM Tris and 100 mM sodium phosphate at various pH levels in the range of 8.0 to 13.0. Additionally, each of these conditions was tested in the presence of 2 M urea, 2 M guanidine hydrochloride, or in the absence of chaotropes. The most effective buffer was determined to be 10 mM Tris with 100 mM sodium phosphate and 2 M urea at pH 12.5 (buffer A, Table 1) based on the low turbidity readings and high yields of soluble protein acquired through its use.

Solubilization and Purification

In order to disaggregate the isolated proteins on a practical scale, an inclusion body pellet equivalent to 0.5 L of bacterial culture was resuspended in 20 mL of buffer A by vortexing. The sample was incubated at room temperature for 30 min to allow for complete solubilization, and then centrifuged at $9,000 \times g$ for 10 min to remove any insoluble components. The clarified sample was diluted 5-fold into water and stirred for 15 min before being added to 20 mL of Ni-NTA His-Bind Superflow resin (QIAGEN, Madison, WI) preequilibrated in 30 mL buffer B. The mixture was stirred for 30 min, then packed into a column and connected to a BioLogic LP chromatography system (Bio-Rad Laboratories, Hercules, CA). The column was washed for 1 hr at a flow rate of 1 mL/min with buffer C and recPrP was then eluted with buffer D at 1 mL/min. Eluate was immediately dialyzed against buffer E, with rapid changes into 4 L of fresh buffer every 30 min for 2.5 hr. Additional purification was achieved through ion-exchange chromatography on a 5-mL CM Sepharose (Sigma) column. After equilibrating the column in buffer E, recPrP was loaded at a flow rate of 0.5 mL/min. Nonspecifically bound contaminants were removed from the column with a 150-mM NaCl wash. To elute recPrP, a gradient from 0.4 M to 1 M NaCl was applied to the column over the course of 50 min at a flow rate of 0.5 mL/min. Fractions containing protein were pooled and dialyzed against five changes of 4 L water to remove salt. Protein can be lyophilized to increase concentration or frozen at -80°C for long-term storage.

Circular Dichroism

A circular dichroism (CD) spectrum for the purified protein was obtained using a Jasco J-715 spectropolarimeter, reading wavelengths from 190 to 270 nm. Cuvettes of 0.2 mm path length were used with recPrP at a concentration of 0.20 mg/mL in water. Scanning 10 nm/min, at a resolution of 2 nm, five spectra were collected and the average of these accumulations was plotted.

Mass Spectroscopy

Matrix-assisted laser-desorption ionization/time-of-flight (MALDI-TOF) mass spectroscopy was used to determine the molecular weight of the purified recPrP. The protein sample was concentrated using an OMIX C4 chromatographic pipette tip (Varian Inc., Santa Clara, CA) and eluted with sinapinic acid matrix onto a Voyager 100-position MALDI sample plate (Applied Biosystems, Foster City, CA). Spectra were acquired using a Voyager-DE Pro Biospectrometry Workstation (Applied Biosystems) with a mass accuracy of ± 10 Da.

Amyloid Fibril Formation

To facilitate conversion of recPrP from its native α -helical form into amyloid fibrils as previously described,^[11,12] protein was brought to a concentration of 0.5 mg/mL in 2 M guanidine, 0.01 M thiourea, and 0.05 M MES buffer, pH 6.0. The solution was incubated for

24 hr at 37°C in a Bioshake iQ thermomixer (Q Instruments, Jena, Germany), shaking at 1700 rpm. Throughout incubation, aliquots were removed and diluted with 10 mM sodium acetate, pH 5.0, to a final PrP concentration of 0.125 mg/mL. Thioflavin T (Sigma) was then added to a final concentration of 260 μ M and the solutions were transferred to a Nunclon Surface 96-well plate (Nunc A/S, Roskilde, Denmark). Thioflavin-T binding was measured on a Tecan Infinite M1000 microplate reader (Tecan Group Ltd., Männedorf, Switzerland), exciting the samples at 445 nm and measuring emissions at 484 nm. Ten measurements were taken for each sample and the average was plotted. Additionally, fluorescence spectra were recorded for samples from the 24-hr time point by exciting at 445 nm and recording emissions in the range of 460 nm to 600 nm. Three spectra were recorded and the average was plotted against the average spectrum from a control sample of identical composition and preparation, but lacking PrP substrate. Data were subjected to a smoothing algorithm fitting the curve to a fourth-order polynomial, averaging 10 neighbors on each side.

RESULTS

During recombinant expression in *E. coli*, recPrP accumulates in bacterial inclusion bodies. In previously described recPrP isolation protocols, a denaturing buffer containing 6–8 M guanidine plus a reducing agent is used to solubilize these inclusion bodies.^[18–20] PrP refolding and disulfide reformation are subsequently achieved by dialysis, a gradient wash step, or dilution in the presence of oxidized glutathione. We investigated whether recPrP could be solubilized from *E. coli* inclusion bodies with lower concentrations of chaotropic agents and no reducing agents, in order to facilitate the retention of native-like protein structure. Our results indicate that alkaline pH facilitates the solubilization of recPrP by either 2 M guanidine or 2 M urea under non-reducing conditions, as judged by a decrease in turbidity and increase in protein concentration of the solubilized supernatant (Figure 1).

Building upon those results, we tested whether solubilization with 2 M urea at pH 12.5 would allow purification of recPrP without a refolding step. A sample of solubilized inclusion bodies was adsorbed to a nickel affinity column, and immediately washed with a buffer containing no chaotropic agents (i.e., no on-column refolding gradient). Elution with imidazole resulted in ~25% yield of the applied recPrP (Table 2 and Figure 2). This yield compares favorably with the ~20% recovery obtained with inclusion bodies solubilized in 6 M guanidine with on-column refolding^[20] or 0% without on-column refolding (data not shown). An additional ion-exchange chromatographic step generated a preparation of recPrP with >95% purity with an appropriate molecular mass of ~23,064 Da, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF mass spectroscopy (Figures 2 and 3).

We used circular dichroism (CD) to examine the secondary protein structure of recPrP solubilized using 2 M urea at pH 12.5 under non-denaturing conditions (and purified without a discrete refolding step). The results indicate that recPrP produced by this method is predominantly α -helical, as judged by the presence of CD minima at wavelengths 210 nm and 218 nm (Figure 4).

To test the functional capacity of our recPrP preparation, we performed an amyloid fibril formation assay monitored by thioflavin T fluorescence. The results show that this recPrP preparation efficiently converts into amyloid in a time-dependent manner (Figure 5A), as confirmed by spectral analysis (Figure 5B).

DISCUSSION

In this article, we report that using a non-reducing buffer containing a relatively low concentration of urea to solubilize recPrP from bacterial inclusion bodies facilitates rapid on-column refolding and subsequent purification, presumably by retaining some native-like secondary structure. Rapid refolding and avoidance of a reduction–oxidation cycle helps to minimize the generation of aggregates, including those containing inappropriate disulfide bonds. The final preparation appears to have appropriate mass, purity, and secondary structure as determined by MALDI-TOF, SDS-PAGE, and CD, respectively. Furthermore, the final preparation readily forms amyloid using a standard fibril formation protocol. Our solubilization and purification procedure provides a yield of 8 mg recPrP/L bacterial culture, which is comparable to existing methods using reducing buffers that contain high concentrations of chaotropes. In summary, we report a convenient alternative to existing protocols for production of α -helical recPrP in which reduction–oxidation cycles are avoided and on-column refolding is achieved rapidly without a chaotrope gradient.

Acknowledgments

We thank James Geoghegan, Michael Miller, and Ashley Zurawel for helpful suggestions and discussion. The National Institutes of Health provided financial support for this study (2R01 NS046478, R01 NS055875, and T32 AI007519).

REFERENCES

1. Prusiner, S.B. Prions Proc. Natl. Acad. Sci. USA. 1998; 95:13363–13383. [PubMed: 9811807]
2. Endo T, Groth D, Prusiner SB, Kobata A. Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. Biochemistry. 1989; 28:8380–8388. [PubMed: 2574992]
3. Loch C, Chesebro B, Race R, Keith JM. Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent. Proc. Natl. Acad. Sci. USA. 1986; 83:6372–6376. [PubMed: 3462700]
4. Stahl N, Borchelt DR, Hsiao K, Prusiner SB. Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell. 1987; 51:229–240. [PubMed: 2444340]
5. Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohen FE, Prusiner SB. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc. Natl. Acad. Sci. USA. 1993; 90:10962–10966. [PubMed: 7902575]
6. Lauren J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature. 2009; 457:1128–1132. [PubMed: 19242475]
7. Donne DG, Viles JH, Groth D, Mehlhorn I, James TL, Cohen FE, Prusiner SB, Wright PE, Dyson HJ. Structure of the recombinant full-length hamster prion protein PrP(29–231): The N terminus is highly flexible. Proc Natl Acad Sci USA. 1997; 94:13452–13457. [PubMed: 9391046]
8. Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wuthrich K. NMR structure of the mouse prion protein domain PrP(121–321). Nature. 1996; 382:180–182. [PubMed: 8700211]
9. Riek R, Hornemann S, Wider G, Glockshuber R, Wuthrich K. NMR characterization of the full-length recombinant murine prion protein, mPrP(23–231). FEBS Lett. 1997; 413:282–288. [PubMed: 9280298]
10. Atarashi R, Moore RA, Sim VL, Hughson AG, Dorward DW, Onwubiko HA, Priola SA, Caughey B. Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein. Nat. Methods. 2007; 4:645–650. [PubMed: 17643109]
11. Baskakov IV, Legname G, Baldwin MA, Prusiner SB, Cohen FE. Pathway complexity of prion protein assembly into amyloid. J. Biol. Chem. 2002; 277:21140–21148. [PubMed: 11912192]
12. Bocharova OV, Breydo L, Parfenov AS, Salnikov VV, Baskakov IV. In vitro conversion of full-length mammalian prion protein produces amyloid form with physical properties of PrP(Sc). J. Mol. Biol. 2005; 346:645–659. [PubMed: 15670611]

13. Breydo L, Makarava N, Baskakov IV. Methods for conversion of prion protein into amyloid fibrils. *Methods Mol. Biol.* 2008; 459:105–115. [PubMed: 18576151]
14. Lu X, Wintrode PL, Surewicz WK. Beta-sheet core of human prion protein amyloid fibrils as determined by hydrogen/deuterium exchange. *Proc. Natl. Acad. Sci. USA.* 2007; 104:1510–1515. [PubMed: 17242357]
15. Nazabal A, Hornemann S, Aguzzi A, Zenobi R. Hydrogen/deuterium exchange mass spectrometry identifies two highly protected regions in recombinant full-length prion protein amyloid fibrils. *J. Mass Spectrom.* 2009; 44:965–977. [PubMed: 19283723]
16. Tycko R, Savtchenko R, Ostapchenko VG, Makarava N, Baskakov IV. The alpha-helical C-terminal domain of full-length recombinant PrP converts to an in-register parallel beta-sheet structure in PrP fibrils: Evidence from solid state nuclear magnetic resonance. *Biochemistry.* 2010; 49(44):9488–9497. [PubMed: 20925423]
17. Wang F, Wang X, Yuan CG, Ma J. Generating a prion with bacterially expressed recombinant prion protein. *Science.* 2010; 327:1132–1135. [PubMed: 20110469]
18. Makarava N, Baskakov IV. Expression and purification of full-length recombinant PrP of high purity. *Methods Mol. Biol.* 2008; 459:131–143. [PubMed: 18576153]
19. Yin SM, Zheng Y, Tien P. On-column purification and refolding of recombinant bovine prion protein: Using its octarepeat sequences as a natural affinity tag. *Protein Express. Purif.* 2003; 32:104–109. 2003.
20. Zahn R, von Schroetter C, Wuthrich K. Human prion proteins expressed in *Escherichia coli* and purified by high-affinity column refolding. *FEBS Lett.* 1997; 417:400–404. 1997. [PubMed: 9409760]
21. Patra AK, Mukhopadhyay R, Mukhija R, Krishnan A, Garg LC, Panda AK. Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coli*. *Protein Express. Purif.* 2000; 18:182–192.

FIGURE 1.

Assay for optimal solubilization conditions. Inclusion body pellets of equal size were resuspended in buffers with different chaotropes and varying pH levels. A562 measures turbidity after a 30-min solubilization and A280 measures protein content in the supernatant after a clarifying centrifugation.

FIGURE 2.

Polyacrylamide gel with aliquots from recPrP purification steps, treated with GelCode Blue Stain Reagent (Pierce, Rockford, IL). Samples include (left to right), solubilized inclusion body (SIB), Ni-NTA column wash (Ni-W), Ni-NTA column eluate (Ni-E), CM column flow-through (CM-FT), CM column wash (CM-W), CM column eluate (CM-E), and a protein ladder (PL) to judge molecular mass based on the relative mobility (M_r) of known markers.

FIGURE 3.

Mass spectrum of the purified protein showing a majority peak at 23,064 Da, congruent with the theoretical molecular weight of recPrP, and a minor peak at 23,279 Da, indicative of sinapinic acid adducts.

FIGURE 4. Circular dichroism spectra of 0.20 mg/mL recPrP with minima at wavelengths of 210 nm and 218 nm.

FIGURE 5.

Conversion of recPrP into amyloid fibrils. (A) Fibril growth curves of recPrP (solid line) and a buffer control (dashed line) as measured by thioflavin-T binding at timepoints from 0 to 24 hr. (B) Thioflavin-T fluorescence spectra when mixed with recPrP (solid line) and a buffer control (dashed line) after 24 hr of shaking under fibril growth conditions. Peak emissions were obtained at 483 nm when excited at 445 nm.

TABLE 1**recPrP Purification Buffers**

Buffer A	10 mM Tris, 100 mM sodium phosphate, 2 M Urea, pH 12.5
Buffer B	2 mM Tris, 20 mM sodium phosphate, 0.4 M Urea, pH 12.0
Buffer C	10 mM Tris, 100 mM sodium phosphate, pH 8.5
Buffer D	10 mM Tris, 100 mM sodium phosphate, 500 mM imidazole, pH 6.0
Buffer E	5 mM Tris, 10 mM sodium phosphate, pH 6.5

TABLE 2

Efficacy and Yield of Purification, with Total Protein Determined by A_{280} and recPrP Measured by Quantification of Gel Code Blue-stained SDS-PAGE Bands

Purification Step	Total Protein (mg)	recPrP (mg)	Yield (%)
Solubilized inclusion body	23.1	16.7	100
Ni-NTA eluate	4.3	4.1	24.6
CM eluate	4.1	4.0	24.0