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Purification of recombinant bovine normal prion protein PrP(104–242) by HPHIC

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Abstract

Purification of the prion protein (PrP) is a major concern for biological or biophysical analysis as are the structural specificities of this protein in relation to infectivity. A simple and efficient method for purification of recombinant bovine normal prion protein containing residues 104–242, PrP(104–242) expressed in *Escherichia coli* by high performance hydrophobic interaction chromatography (HPHIC) was presented in this work. The solution containing denatured and reduced protein in 8.0 mol/L urea extracted from the inclusion body was directly injected into the HPHIC column, aggregates were prevented by the interaction between the denatured PrP(104–242) molecules and the stationary phase during the chromatographic process, the soluble form of PrP(104–242) in aqueous solution was obtained after desorbed from the column. Several factors, including pH value, types of stationary phase and salt, and gradient mode, influencing the purification results were investigated. Optimal conditions were obtained for the purification of PrP(104–242) by HPHIC. This procedure yield PrP(104–242) of a purity of 96% with a recovery of 87%, respectively, for a single step purification of 40 min.

Keywords: Protein refolding; Bovine normal prion protein

1. Introduction

Investigation of prion protein (PrP) has gained broad interest following the explosion of bovine spongiform encephalopathy (BSE) in England and the Nobel Prize award to professor Prusiner on the discovery of prion protein [1]. The prion diseases in animals include scrapie of sheep and goats as well as BSE, whereas infective forms prions in human are presented by Creutzfeldt–Jakob diseases (CJD), Gerstmann–Straussler–Scheinker syndrome (GSS), and fatal familial insomnia (FFI) [2–4]. The human prion diseases are unique in that they can present as sporadic, inherited, or infectious [5]. The transmissible pathogens causing the prion deseases are composed largely, if not entirely, of an abnormal isoform of prion protein (PrP) designated PrP^{Sc} [6].

There are two isoforms of PrP, one is PrP^{C} and the other is PrP^{Sc}. To study the structures of the PrP isoforms, large quantities of purified recombinant PrP (rPrP) are required. Mehlhorn and coworker expressed Syrian hamster (Sha) PrP in *Escherichia coli*, after solubilization of rPrP in 8 mol/L GdnHCl, it was purified by size exclusion chromatography (SEC) and reversed phase liquid chromatography (RPLC). This procedure yielded a recovery of approximately 50% [7]. Rezaei and coworker developed a high yield one-step method for the purification of the full-length recombinant sheep PrP, based on the affinity of the conserved octapeptide repeats for transition-metal cation [8]. Negro described the high-level expression in *E. coli*, and purification in the monomeric form by affinity chromatography, using a histidine-tagged full-length mature PrP of bovine brain, termed His-PrP [9]. Zahn presented an efficient method for the expression of intact mammalian prion proteins as histidine tail fusion proteins into inclusion bodies in the cytoplasm of *E. coli*. The recombinant His-tagged PrP was

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refolded and oxidized while N-terminally immobilized on a nickel-NTA agarose resin. This 'high affinity column refolding' facilitates the preparation of prion proteins by preventing protein aggregation and intermolecular disulfide formation. After elution from the resin the histidine tail can be removed using thrombin without cleaving the prion protein polypeptide chain [10]. Most of the methods reported on the purification of the $rPrP^C$ used affinity chromatography (AFC) or a combination of several chromatography techniques. AFC purification often result in high cost and short column life which limit its wide application. Also, PrP^C need to be expressed as a fusion protein which must be digested and purified to obtain the final product. The combination of several chromatography techniques can often results in poor recovery yield of the PrP.

Therefore, a simple and efficient method to purify PrP is necessary to study its structure and function. High performance hydrophobic interaction chromatography (HPHIC) is applied extensively to the purification of proteins due to its high activity recovery and low cost of its mobile and stationary phases [11]. Geng and Chang were first to present protein refolding by HPHIC [12], and applied this method to the refolding of several proteins [12–16]. Geng and Bai recently elucidated the mechanism of protein refolding by HPHIC in details [17]. Wang et al. cloned a fragment containing residues 104–242, PrP(104–242), and full length of recombinant bovine normal prion protein, PrP(24–242), and expressed them in *E. coli* [18]. Previously, PrP(24–242) was purified by HPHIC with good results [19]. Here, we describe the purification of the PrP(104–242) by HPHIC with high purity and high yield. This should facilitate studies designed to determine the multiple tertiary structures of PrP.

2. Experimental

2.1. Equipment

An LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of two pumps (LC-6A), a variable-wavelength UV-Vis detector (SPD-6AV), a system controller (SCL-6B) and a recorder (Dahua Instrument Co., Shanghai, China) was used. Stainless-steel column (100 mm × 4.0 mm i.d.) was packed with a packing apparatus (Xizhu Center of Technology Service, Beijing, China) by slurry-packing method. A Biologic LP liquid chromatograph (Bio-Rad, Sweden) was used for desalting sample using SEC column. Three types of HPHIC packings containing end groups of PEG600, phenyl and tetrahydrofuryl alcohol (THFA), respectively, used were all made in our institute according to reference [20]. A 5 L fermentor (Braun, Germany) was used to express protein. A UV spectrometer (Third Analytical Instrument Co., Shanghai, China) and a dual-wavelength thin layer chromatographic scanner (CS-930, Shimadzu) were used for the determination of the total amount of proteins and verify the purity of the PrP(104–242), respectively. A SORVALL RC28S centrifuge (Kendro Laboratory Products) was used.

2.2. Chemicals

Standard protein markers were purchased from Institute of Biochemicals (Shanghai, China). BCA Protein Assay Kit was obtained from PIERCE Co. (Rockford). Other chemicals used are of analytical-grade. Deionized water (Barnstead E-pure) was used.

2.3. Expression

Expression was conducted in our institute according to the reference [21]. Cultures were grown at 37 °C in LB medium supplemented with kanamycin (100 μg/mL) in 5 L fermentor, and induced at a cell density of 0.5 (A_{600} nm) by the addition of 0.001 mol/L isopropyl-thiogalactoside (IPTG). Once cells reached an optical density of 1.2, they were harvested by centrifugation at 6000 rpm and 4 °C for 15 min, and frozen at −20 °C. Forty-two grams of wet biomass were obtained finally.

2.4. Recovery of PrP(104–242) inclusion bodies

The cells were thawed at room temperature and washed with a buffer of sodium phosphate (0.02 mol/L, pH 7.2) containing 0.001 mol/L EDTA, and then the suspension was centrifuged at 6000 rpm and 4 °C for 15 min after washing. The supernatant was discarded, and this procedure was repeated once. The cells were frozen at −20 °C for 24 h. Twenty grams of the frozen cells were thawed at room temperature and resuspended in 200 mL of a cold buffer of 0.02 mol/L sodium phosphate (pH 7.2) containing 0.001 mol/L EDTA. The cells were lysed by sonication on ice. The lysates were centrifuged at 18,000 rpm for 15 min to collect the insoluble protein aggregates. The pellet (protein aggregates and cell debris) was washed twice with 30 mL of 0.1 mol/L Tris–HCl (pH 8.0) including 4.0 mol/L urea, 0.2 mol/L NaCl and 0.02 mol/L EDTA. The pellet was subsequently washed with 30 mL of 1.0 mol/L NaCl. After each washing step, the suspension was centrifuged at 18,000 rpm and 4 °C for 15 min. About 2.4 g of pellet fraction containing PrP(104–242) inclusion bodies was obtained and stored at −20 °C prior to further separation.

2.5. Solubilization of PrP(104–242) inclusion bodies

After thawing, 1.0 g of PrP(104–242) inclusion bodies were resuspended in 20 mL of solubilization buffer composed of 8.0 mol/L urea, 0.1 mol/L Tris–HCl (pH 8.0), 0.2 mol/L NaCl, 0.1 mol/L β-mercaptoethanol, 20 mmol/L EDTA. After overnight incubation at 4 °C, the solution was centrifuged at 18,000 rpm and 4 °C for 15 min, the supernatant containing PrP(104–242) was stored at 4 °C. The to-

tal protein concentration in the supernatant was determined to be 8.0 mg/mL by BCA Protein Assay Kit. The purity of PrP(104–242) in the inclusion body was measured to be 14% by SDS–PAGE and thin-layer chromatographic scanner. Through calculation, about 115 mg of recombinant PrP(104–242) could be obtained from the expression system.

2.6. High performance hydrophobic interaction chromatography

PrP(104–242) was purified by HPHIC on a prepacked column. The column liquid chromatography was performed at room temperature on a HPLC system. The PrP(104–242) extract was centrifuged without further treatment at 18,000 rpm and 4 °C for 15 min prior to sample loading. One millilitre of the sample solution containing 8.1 mg of total protein was directly injected into the HPHIC column previously equilibrated with 80% solution A (3.0 mol/L ammonium sulfate containing 0.05 mol/L phosphate, pH 7.0) and 20% solution B (0.05 mol/L phosphate, pH 7.0). The protein desorption was performed using a nonlinear gradient elution in 40 min as in the Fig. 1c. HPHIC flow rate was 1.0 mL/min at UV detection was set at 280 nm. Protein extracts were separated by SDS–PAGE. Appropriate fractions were pooled and their protein concentrations were estimated by the BCA Protein Assay Kit.

2.7. Desalting

Ten milliliters of the collected fraction from HPHIC was injected into the size exclusion chromatography (SEC) column (200 mm × 20 mm i.d.) prepacked with G-25 packings (Pharmacia, Sweden), which previously equilibrated with 0.02 mol/L sodium phosphate (pH 7.0) containing 0.1 mol/L NaCl at a flow-rate of 3.0 mL/min.

2.8. Electrophoresis

Sodium docecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed under reducing conditions with 15% gels using the Bio-Rad Mini-PROTEIN II electrophoresis unit. The collected fractions from the column were precipitated by 10% volume of 100% trichloroacetic acid (m/v). The deposit was resolved with Laemmli buffer and the samples were treated in denaturing buffer in boiling water for 3–5 min before being loaded. The SDS low-molecular-weight standard mixture was used in order to determine the apparent molecular weight of the samples. Protein bands were visualized by silver staining.

2.9. Circular dichroism (CD) spectroscopy

CD spectra was recorded at room temperature using a spectropolarimeter (Jasco, model 715, Tokio, Japan). Proteins were dissolved in 10 mmol/L sodium phosphate buffer

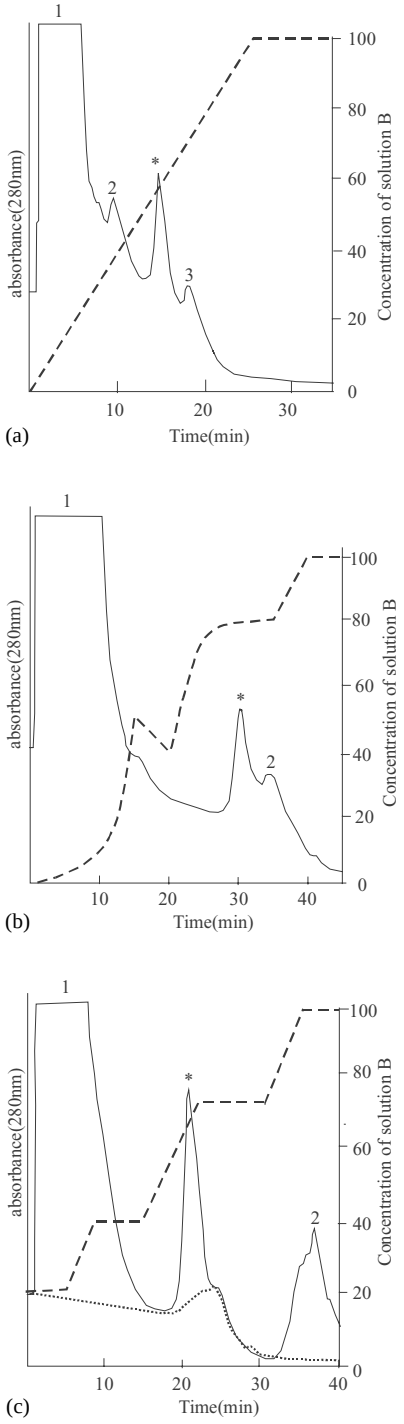

Fig. 1. Chromatograms of PrP(104–242) by different gradients. Conditions: The column (100.0 mm × 4.0 mm i.d) packing with HIC packings with phenyl end group; moble phase A: 3.0 mol/L ammonium sulfate containing 0.05 mol/L phosphate, pH 7.0; mobile phase B: 0.05 mol/L phosphate, pH 7.0; eluted with different gradients (a, linear gradient in 25 min from 100%A to 100%B, delay 10 min. (b) 0.01 min, 0%B; 1 min, B curve = −2; 15 min, 50%B; 20 min, 45%B; 20 min, B curve = 3; 35 min, 80%B; 40–45 min, 100%B. (c) 0–5 min, 20%B; 8–15 min, 40%B; 23–30 min, 70%B; 35–40, 100%B.) (*) PrP(104–242); (1) solvent; (2 and 3) impurities. The solid lines are elution profiles during purification, dash lines are gradient profiles, and dot lines is blank gradient elution profile.

(pH 7.0). A 0.01 cm path length quartz cells was used to record spectra of proteins in the far ultraviolet region (190–250 nm) at protein concentration of 0.2 mg/mL. CD spectra were acquired at a scan speed of 20 nm/min, 1 nm bandwidth, and a response time of 1 s. The sample compartment and the sample solution were purged with dry nitrogen gas.

3. Results and discussion

The main factors affecting the separation of proteins using HPHIC are usually the type of ligand and matrix, salt composition and pH, temperature, additives, and gradient mode [22].Below we describe the influence of these parameters on the separation of the PrP(104–242).

3.1. Type of salt

During HPHIC, the solvent-accessible hydrophobic regions on the surface of biomolecules interact with the hydrophobic ligands on the adsorbent. From the retention mechanism of proteins in HPHIC, this adsorption is salt-promoted so that the binding of proteins with the stationary phase is usually carried out at high salt concentrations. Elution and separation, according to differences in the surface hydrophobic of proteins [23], are in general brought about by decreasing the salt concentration in the mobile phase [24]. In order to determine the influence of salts on the selectivities and resolution of the PrP(104–242) we carried out the separation using elution of the following salts: 5.0 mol/L NH_4Cl, 5.0 mol/L NaCl, 5.0 mol/L CH_3COONH_4, 1.0 mol/L Na_2SO_4 and 3.0 mol/L $(NH_4)_2SO_4$ as eluent A. Optimal separation conditions were obtained by using 3.0 mol/L $(NH_4)_2SO_4$ as eluent A.

3.2. Stationary phases

For HPHIC, the nature of stationary phase can have an important influence on the protein separation. In this experiment, three types of stationary phases with end groups of phenyl, PEG600 and tetrahydrofurfuryl alcohol (THFA) were investigated. The fractions of PrP(104–242) were collected and separated on SDS–PAGE. The purity and mass recovery of PrP(104–242) obtained with the three types of column were shown in Table 1. As can be seen from Table 1, the purity and mass recovery of the purified PrP(104–242) by the phenyl column was higher than that obtained by both THFA and PEG600 columns.

3.3. pH

Enhancement of chromatographic selectivity can be obtained by manipulating the pH of the eluent [25]. For the investigation of pH effects in our system, the phosphate buffer concentration was 0.05 mol/L for all pH values and 3.0 mol/L ammonium sulphate was used in the eluent A. It was found that the pH effects on the separation did not show any change in selectivity and resolution in the pH range examined. However, PrP(104–242) is in the most stable configuration when pH is 7.0, and was selected in this study.

Table 1
Purity and mass recovery of PrP(104–242) obtained with three types of column

Column	Purity (%)	Mass recovery (%)
PEG600	79	71
THFA	63	52
phenyl	88	89

The columns (100.0 mm × 4.0 mm i.d.) packing with HIC packings with PEG600 (a), THFA (b) and phenyl (c) end groups, respectively. The linear gradient elution was from 100% solution A (3.0 mol/L ammonium sulfate containing 0.05 mol/L phosphate, pH 7.0) to 100% solution B (0.05 mol/L phosphate, pH 7.0) at a flow rate of 1.0 mL/min for 30 min with a delay for 10 min.

3.4. Gradient mode

As shown in Fig. 1, slight variations in the gradient mode resulted in a significant change in resolution. Three different gradient modes, linear gradient (Fig. 1a), nonlinear gradient containing curvatures (Fig. 1b), nonlinear gradient not containing curvatures (Fig. 1c) were employed for this investigation. As can be seen from this figure, the separation of PrP(104–242) and impurities using the nonlinear gradient not containing curvatures (Fig. 1c) is the best among the three gradient modes used in the present work. The fractions of PrP(104–242) by the three gradients were collected and separated on SDS–PAGE. The purity of PrP(104–242) purified by gradient in Fig. 1a is 85%, Fig. 1b is 77%, Fig. 1c is 96%, respectively.

Considering the effect of each factor, the conditions selected for the purification of PrP(104–242) were: phenyl column as the stationary phase, companying with 3.0 mol/L $(NH_4)_2SO_4$ (pH 7.0) containing 0.05 mol/L phosphate as eluent A, and 0.05 mol/L phosphate buffer (pH 7.0) as eluent B, under the nonlinear gradient shown in Fig. 1c would be the optimized chromatographic condition for the purification of PrP(104–242) by HPHIC.

3.5. The purity and mass recovery of PrP(104–242)

Fig. 2 shows the SDS–PAGE of PrP(104–242) purified by HPHIC under the conditions of Fig. 1c. As can be seen from the figure, PrP(104–242) purified by HPHIC was in the same position as the PrP(104–242) in the inclusion body, its molecular weight was about 18,000 Da. The purity of the PrP(104–242) in the inclusion body was measured to be only about 14% by dual-wavelength thin-layer chromatographic scanner (not shown), but the purity of PrP(104–242) purified by HPHIC from 8.1 mg of total protein was about 96%, and its mass recovery was determined to be 87%, 0.98 mg of pure PrP(104–242) was obtained by one-step purification.

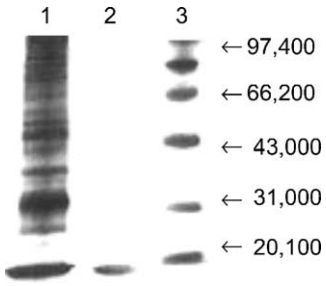

Fig. 2. Electrophoregram of PrP(104–242) purified HIC. (1) PrP(104–242) inclusion body extract, (2) PrP(104–242) purified by HIC with the gradient of Fig. 1c, and (3) molecular weight marker.

3.6. CD spectroscopic characterization of PrP(104–242)

The secondary structure of the isolated recombinant PrP(104–242) was assessed by CD analysis where the double ellipticity minimum was at approximately 208 and 222 nm (Fig. 3), which indicates a high content of α-helical secondary structure and a low amount of β-sheet structure. Therefore, the purified PrP(104–242) have a spatial structure close or identical to the native PrP(104–242) protein. The result showed that PrP(104–242) was purified and simultaneously refolded by one step of HPHIC.

The purification protocol presented here has some novelties when comparing to the earlier investigations. Mehlhorn et al. [7] described a method to purify Syrian hamster PrP using SEC combining with RPLC to obtain a mass recovery about 50% due to two-step purification. Zahn developed an efficient method for the purification of human prion protein [10]. Prion proteins were expressed as histidine tail fusion proteins into inclusion bodies in the cytoplasm of *E. coli*. The recombinant His-tagged PrP was refolded and oxidized while N-terminally immobilized on a nickel-nitrilotriacetic acid (NTA) agarose resin. This 'high affinity column refolding' facilitates the preparation of prion proteins by preventing protein aggregation and intermolecular disulfide formation. But the histidine tail must be removed using thrombin after elution from the resin, and a step of ion exchange chromatography must be used to remove thrombin, the cleaved tail need be removed by dialysis against water, these steps make the method relatively complex and inconvenient.

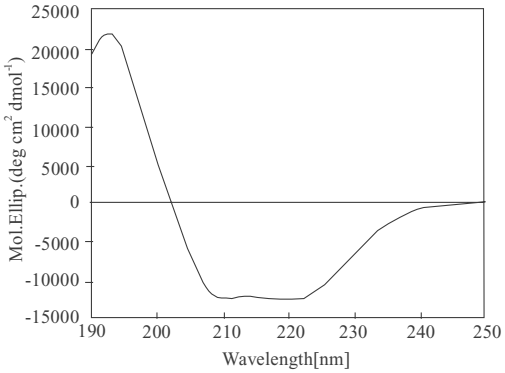

Fig. 3. Far UV CD spectra of purified PrP(104–242). CD spectra were recorded at room temperature using a spectropolarimeter (Jasco, model 715, Tokio, Japan). Proteins were dissolved in 10 mmol/L sodium phosphate buffer (pH 7.0). A 0.01 cm path length quartz cells was used to record spectra of proteins in the far ultraviolet region (190–250 nm) at a protein concentration of 0.2 mg/mL. CD spectra were acquired at a scan speed of 20 nm/min, 1 nm bandwidth, and a response time of 1 s. The sample compartment and the sample solution were purged with dry nitrogen gas.

In the presented work, when the denatured/reduced form of PrP(104–242) was injected into the HPHIC column, and the denaturants was removed rapidly and completely. PrP(104–242) is adsorbed on the stationary phase of hydrophobic interaction chromatography at a high concentration of ammonium sulfate, and the hydrophobic interactions between the denatured PrP(104–242) molecules and the stationary phase can prevent the denatured protein molecules from aggregating. By decreasing the concentration of ammonium sulfate, the denatured PrP(104–242) refolded gradually and then desorbed at low concentration of ammonium sulfate to refold into its native state. PrP(104–242) can be purified and simultaneously refolded by one HPHIC run. Compared with the procedures developed by Mehlhorn and coworkers [7,10], the presented work has higher mass recovery of PrP(104–242) and is very simple. In addition, PrP doesn't require to be expressed as a fusion protein, and steps to remove the histidine tail and protease are not necessary.

The present method can be scaled up by increasing the dimensions of HPHIC column to support the purification of large amounts of PrP(104–242) required for the characterization of the biochemical and biophysical properties [13].

4. Conclusions

A simple and rapid method for the purification with simultaneous refolding of PrP(104–242) from inclusion bodies using a single hydrophobic interaction chromatography (HIC) is presented in this report. The interactions between the denatured PrP(104–242) and the stationary phase of HIC prevented the denatured PrP(104–242) molecules from aggregation, thus providing higher mass recovery. The purified PrP(104–242) have high content of α-helical secondary structure. With the optimized chromatographic condition, both purity and mass recovery of the purified PrP(104–242) were found to be more than 90% only by one step with HIC in 40 min. This method would be employed for the fast preparation of sufficient amounts of PrP(104–242) required for the study the structure of PrP(104–242).

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