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Evaluation of non-immunoaffinity methods for isolation of cellular prion protein from bovine brain

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ABSTRACT

Transmissible spongiform encephalopathies (TSEs) are progressive neurodegenerative diseases that affect the central nervous system of many animals, including humans. Research suggests that TSEs are caused by conversion of the cellular prion protein (PrP^C), which is encoded in many tissues, especially brain, to the pathological form (PrP^{Sc}). This conversion affects PrP^{Sc} structure, conferring different biochemical properties, such as the increased resistance to proteinase K, that have been widely used for its purification. By contrast, PrP^C is less resistant and its isolation is more challenging. Here, we propose a purification strategy to efficiently recover PrP^C from healthy bovine brain using conventional non-immunoaffinity methods. The applicability of extraction using detergents, size exclusion chromatography, diafiltration with molecular weight cutoff (MWCO) filters, and immobilized metal affinity chromatography (IMAC) using Western blot (WB) analysis to detect the presence of PrP^C is discussed in detail.

Transmissible spongiform encephalopathies (TSEs)[1] belong to a group of progressive neurodegenerative diseases that affect the central nervous system in animals and humans such as scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and Creutzfeldt–Jakob disease (CJD) and fatal familial insomnia (FFI) in humans [1]. The causative agent of TSEs is believed to be linked to the presence of a host-encoded prion protein (PrP), which is expressed mostly in the brain [1,2]. The conformational transition from the native cellular prion protein (PrP^C) to the disease-related abnormal scrapie isoform (PrP^{Sc}) has been proposed as the critical event in TSE pathogenesis [1,2]. This posttranslational conversion involves noncovalent modifications, leading to aggregation of PrP^{Sc} into scrapie-associated fibrils, which form the typical proteinaceous plaques. The appearance of these plaques and the spongiform modifications of the gray matter are the main histopathological features of TSEs [3]. PrP^C contains two *N*-linked glycosylation sites and is a glycosylphosphatidylinositol (GPI)-anchored membrane protein with a higher content of α-helices than β-sheets in the secondary structure [2,4–14]. In contrast to PrP^C, PrP^{Sc} is partially proteinase K resistant, is insoluble in nonionic detergents, and does not bind to copper [9]. These specific biochemical properties have been widely used for differentiation between PrP^{Sc} and PrP^C, facilitating purification and detection of PrP^{Sc} as well as diagnosis of TSEs [5]. In contrast, isolation of native PrP^C from brain tissue is more challenging, and many authors have preferred to use recombinant prion protein (rPrP) expressed in *Escherichia coli* as standard because it is easier to obtain and purify with large recoveries [2,6,7,10,15–17]. However, rPrP is unlikely to be truly representative of PrP^C because the recombinant protein expressed in *E. coli* lacks the posttranslational modifications of the native mammalian protein [2,7,10,11]. Therefore, developing efficient methods for large-scale purification of PrP^C in its natively folded form with all of its posttranslational modifications is critical to obtain authentic standards to compare with expressed products.

In general, methods for isolation of PrP^C from brain include a first step of preparation of brain homogenates and subcellular fractionation by differential centrifugation to obtain the membrane fraction. Then, PrP^C is separated from the membrane with the use of detergents or a specific lipase called phosphatidylinositol-specific phospholipase C (PI–PLC). Soluble fractions containing PrP^C are pooled and purified by immunoprecipitation [18], diafiltration

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[1] *Abbreviations used:* TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy; PrP, prion protein; PrP^C, cellular prion protein; PrP^{Sc}, scrapie isoform of prion protein; rPrP, recombinant prion protein; MWCO, molecular weight cutoff; IMAC, immobilized metal affinity chromatography; IAC, immunoaffinity chromatography; SEC, size exclusion chromatography; WB, Western blot; MALDI–TOF–MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; TFA, trifluoroacetic acid; CA, carbonic anhydrase; RNase A, ribonuclease A; BSA, bovine serum albumin; ALB, albumin; SOD-1, superoxide dismutase 1; EDTA, ethylenediaminetetraacetic acid; MP, mobile phase; zwittergent 3-12, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate.

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using molecular weight cutoff (MWCO) filters [7], or chromatographic techniques such as cationic exchange chromatography [2,12,19], immobilized Cu^{2+} or Co^{2+} affinity chromatography (Cu^{2+}– or Co^{2+}–IMAC) [2,3,7,12,20], immunoaffinity chromatography (IAC) [19], lectin affinity chromatography [21], and size exclusion chromatography (SEC) [20,22]. Here, we describe a procedure that does not include immunoaffinity-based steps, which are the "gold standard" proposed by others due to the excellent selectivity [23,24]. However, immunoprecipitation and IAC are difficult to scale up due to the elevated costs, poor recoveries, and limited stability of anti-PrP^C sorbents [12]. In our method, the PrP^C extracted from the membrane fraction obtained by centrifugal filtration of the brain homogenates is purified using SEC, followed by diafiltration and Cu^{2+}–IMAC, and Western blot (WB) analysis is applied for PrP^C detection. The purity of the final product is demonstrated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS).

Materials and methods

Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Acetone, acetonitrile, methanol, ethanol, hydrochloric acid (25%), trifluoroacetic acid (TFA), ammonia (25%), and copper(II) sulfate pentahydrate were supplied by Merck (Darmstadt, Germany). Carbonic anhydrase (CA) from bovine erythrocytes and imidazole were purchased from Fluka (Madrid, Spain). Potassium chloride, sodium chloride, sinapinic acid, ribonuclease A (RNase A) from bovine pancreas, dl-dithiothreitol (for electrophoresis), bovine serum albumin (BSA), albumin from chicken egg white (ALB), superoxide dismutase 1 (SOD-1) from bovine erythrocytes, sodium bicarbonate, sucrose, Tween 20, and *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate were supplied by Sigma (Madrid, Spain). Tris(hydroxymethyl)aminomethane and ethylenediaminetetraacetic acid (EDTA) disodium salt were purchased from J. T. Baker (Deventer, The Netherlands) and Panreac (Barcelona, Spain), respectively. Aprotinin from bovine lung, leupeptin hemisulfate, and pepstatin A were supplied by Calbiochem (Darmstadt, Germany). Water with a conductivity value lower than 0.05 μS/cm was obtained using a Milli-Q water purification system (Millipore, Molsheim, France).

Protein standard solutions and samples

Individual solutions (0.5 mg ml^{-1}) and a mixture of BSA, ALB, CA, and RNase A were prepared in one of the mobile phases (MPs) tested in SEC (10 mM Tris and 0.15 M KCl, pH 7.5, SEC MP-2). A 1-mg ml^{-1} solution of commercial SOD-1, which is a homodimer formed by two identical monomers that coordinates 1 Cu^{2+} and Zn^{2+} per unit (Cu_2, Zn_2-dimer SOD-1) [25], was demetalated to obtain Apo–SOD-1 by acidification with TFA and diafiltration following a procedure described elsewhere [26]. The final Apo–SOD-1 solution of approximately 1 mg ml^{-1} was dissolved in SEC MP-2 and used as a standard for Cu^{2+}–IMAC.

The brain of a healthy 4-year-old Pyrenean Brown female cow tested negative for BSE was provided by the Animal Tissue Bank of Catalunya (BTAC) of the Autonomous University of Barcelona (Bellaterra, Spain). It was cut into slices and stored at −80 °C until its use.

Extraction

PrP^C was extracted from the membrane fraction, combining and adapting different parts of several methods described in the literature [2,3,7,12,19,20,22,27,28].

To hinder denaturation and inactivation processes, all steps were performed at 4 °C. After thawing, approximately 40 g of midbrain was homogenized with a Polytron PT-10-35 (Kinematica, Lucerne, Switzerland) for three periods of 5 s separated by a 10-s break in an Erlenmeyer flask containing 200 ml of ice-cold homogenization buffer (10 mM Tris, 100 mM KCl, 320 mM sucrose, 1 mM dithiothreitol, 1 mM $NaHCO_3$, 5 μg·ml^{-1} leupeptin, and 10 μg ml^{-1} aprotinin, pH 7.5). To collect the membrane fraction, the homogenates were centrifuged at 5500 rpm for 10 min at 4 °C (Avanti J-25 centrifuge, Beckman Coulter; rotor: JA 25.5). The pellet was removed and saved, and the resulting supernatant was centrifuged at 10,000 rpm for 10 min. The new pellet was collected and mixed with the previous one. Both pellets were solubilized in 60 ml of incubation solution (1% [w/v] *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate [zwittergent 3-12], 5 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, and 1 μg ml^{-1} pepstatin) using mechanic agitation for 90 min. Then, 90 ml of dilution buffer (10 mM Tris, 100 mM KCl, 5 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, and 1 μg ml^{-1} pepstatin, pH 7.5) was added to make a final concentration of 0.4% (w/v) *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate. The suspension was stirred overnight and later centrifuged at 30,000 rpm for 3 h at 4 °C (L-90 K ultracentrifuge, Beckman Coulter; rotor: 35). The final supernatant was separated and filtered through 0.45-μm, followed by 0.22-μm, nylon filters (MSI).

Purification

SEC

Before SEC, the filtered extract was concentrated 5 times using Amicon Ultra-10 centrifugal devices (MWCO = 10 kDa) (Millipore, Beverly, MA, USA) at 4100 rpm in a Rotanta 460 centrifuge at 4 °C (Hettich Zentrifugen, Tuttlingen, Germany).

SEC was performed with a HiLoad 16/60 Superdex 75 prep-grade column (GE Healthcare, Uppsala, Sweden) using an HP 1100 series liquid chromatograph with a diode array detector (Agilent Technologies, Waldbronn, Germany). Instrument control, data acquisition, and data processing were performed using Chemstation LC3D software (Agilent Technologies). With the pressure of this semipreparative column as a limiting factor, the flow rate was set up to the highest possible (1.5 ml min^{-1}) to achieve a reasonable separation time (90 min/run). The column was first equilibrated with 1 column volume of 10 mM Tris and 0.015 M KCl (pH 7.5) (SEC MP-1) and then with 4 column volumes of 10 mM Tris and 0.15 M KCl (pH 7.5) (SEC MP-2). Absorbance was monitored at 220 nm. A calibration curve was constructed by injecting 1 ml of the mixture of protein standards, after identification using individual solutions. The concentrated extract was fractionated injecting volumes of 1 ml and using an LKB Bromma 2212 Helirac fraction collector (LKB, Bromma, Sweden). Fractions were collected for convenience, in accordance with the observed chromatogram, at 7-min intervals after 2 min of the injection. Fractions potentially containing proteins were filtered through 0.45-μm nylon filters (MSI) and concentrated 15 times using Amicon Ultra-10 centrifugal devices (MWCO = 10 kDa) as indicated at the beginning of this subsection.

Diafiltration using MWCO filters

A single step of diafiltration using different MWCO filters was tested as an alternative to purification with SEC. Here, 15 ml of the filtered extract was centrifuged using Amicon Ultra-30, −50, or −100 centrifugal devices (MWCOs = 30, 50, or 100 kDa, respectively) (Millipore) until reducing the volume to 3 ml (4100 rpm, 4 °C, Rotanta 460 centrifuge).

IMAC

IMAC was performed with a HiTrap IMAC HP (1 ml) and Hi Prep IMAC FF 16/10 (20 ml) columns (GE Healthcare) using an HP 1100