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Establishment of Cell Free Conversion System With Biotin-labelled Recombinant PrP^{sen} Expressed in *E. coli*[1]

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Objective To report a protocol using biotin-labelled PrP protein in cell free conversion assay instead of isotope. **Methods** A hamster PrP protein (HaPrP) was expressed in *E. coli* and purified with HIS-tag affinity chromatograph. After being labelled with biotin, HaPrP was mixed with PrP^{Sc} preparation from scrapie strain 263K. **Results** Protease-resistant bands were detected after four-day incubation. **Conclusion** The new conversion model provides a reliable, easily handling, and environment-friendly method for studies of prion and transmissible spongiform encephalopathies.

Key words: Transmissible spongiform encephalopathies; Prion; Biotin; Cell free conversion; *E. coli*

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), or prion diseases are rare fatal neurodegenerative diseases of human beings and other animals. One hallmark of these diseases is the presence of a disease-specific protease-resistant form of PrP (PrP^{Sc} or PrP^{res}) in central nerve tissues[1]. The unique properties of the infectious agents are proteinaceous and devoid of nucleic acid, with aggregative and amyloidogenic characteristics. The essential threshold of prion disease is that the pathological and infectious isoform PrP^{Sc} can induce the conversion of the normal protease-sensitive form of the host's prion protein (PrP^{C} or PrP^{sen}) to the abnormal form[2]. Although the exact mechanism of conversion is still unknown, there is evidence that the interaction between PrP^{C} and PrP^{Sc} is required for the formation of PrP^{Sc} and TSE pathogenesis[3].

In the cell free system, PrP^{Sc} isolated from the brains of infected animals can induce the conversion of PrP^{C} that is usually isotope-labelled to a proteinase K (PK)-resistant isoform. The newly formed PK-resistant PrP protein can be distinguished from the input PrP^{Sc} in radioautography[4]. Usually, the isotope-labelled recombinant PrP^{C} purified from mammalian cells[5-8] or from baculovirus-infected insect cells is used in the cell free conversion assay[9-10]. Although radioactivity is considered to be somewhat sensitive for detection of nascent PrP^{res}, the limited half-life, the relatively low recovery of labelled PrP^{C}, as well as the complicated safety protection procedures for isotope are unsatisfactory. In this report we describe a method using a biotin-labelled recombinant hamster PrP^{C} expressed in *E. coli*, instead of commonly used ^{35}S-methionine-labelled protein, in the cell free conversion assay.

MATERIALS AND METHODS

Expression and Purification of Hamster PrP

The nucleotide sequence coding for hamster PrP (aa 23-231) was released from a previously constructed plasmid pT-HaPrP[10] with Bam HⅠ and Sal Ⅰ and subcloned into the plasmid pQE30 (Qiagen), generating plasmid pQE-HaPrP23-231. The recombinant plasmid was transformed into *E. coli* strain M15 and

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the expression of recombinant protein was induced by 1 mmol/L IPTG. After sonication and centrifugation of the 6 mol/L GdnHCl denatured *E. coli* cell lysate, the fraction of soluble protein was added into a nickel-NTA agarose resin (Qiagen). The column was washed and eluted under denaturing conditions in 8 mol/L urea by gradual pH elution. Denatured protein was renatured by simple dialysis against water and lyophilized and stored at -80℃. Protein concentration was determined with a commercially supplied BCA protein kit (Beyotime).

Western Blot

To verify the immunoreactivity, the protein was separated and further transferred onto nitrocellulose membrane by electroblotting. The membranes were incubated with PrP-specific monoclonal antibody 3F4 (Dako) for 2 h at room temperature and the signals were visualized with horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Santa Cruz).

Biotin Labelling of Hamster PrP

To label the HaPrP with biotin, the lyophilized HaPrP was dissolved in PBS to 1 mg/mL and labelling process was carried out according to the instructions of biotin labelling kit (Roche). Briefly, 1 mg purified HaPrP in 1 mL volume was mixed with 7 μL of 20 mg/mL biotin-7-NHS in DMSO. After the mixture was incubated for 2 h at room temperature with gentle stirring, the excess labelling reagent was separated by gel-filtration on a prepared Sephadex G-25 column. The biotin-labeled HaPrP was separated in 12% SDS-PAGE, and transferred onto nitrocellulose membrane. The efficiency of biotin labelling was calculated by incubating with HRP conjugated streptavidin and developed in DAB substrate.

Preparation of PrP^{Sc}

PrP^{Sc}-enriched fractions were prepared from the brains of Syrian hamsters infected with scrapie strain 263K as described[11-14]. In brief, PrP^{Sc} was purified in the absence of protease inhibitors by centrifugation at 2000rpm for 10 min from 10% (w/v) homogenized brain in lysis buffer (10 mmol/L NaCl, 10 mmol/L EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 10 mmol/L Tris, pH 7.4). The supernatants were collected and centrifuged at 20 000 g for 90 min, and the pellets were collected and stored at -20℃. The presence of PK-resistant PrP^{Sc} in the preparations was confirmed by PK-treated Western blot as described elsewhere[12-14].

Quantitative analysis of the immunoblot images was carried out using computer-assisted software Image Total Tech (Pharmacia). Briefly, the image of immunoblot was scanned with Typhoon (Pharmacia) and digitalized. The values of each target blot were evaluated and balanced with those of a genetic engineering-expressed HaPrP (recombinant hamster's prion protein) used as a control standard.

Cell Free Conversion

To prepare the cell free conversion system for PrP protein *in vitro*, the extracted PrP^{Sc} was partially denatured with 2.5 mol/L GdnHCl in 2 X conversion buffer (0.1 mol/L sodium citrate, pH 6.0, 2% N-laurylsarcosine, and 10 mmol/L cetylpyridinium chloride) at 37℃ for 24 h. An aliquot of treated PrP^{Sc} preparation roughly equal to 26 μg PrP^{Sc} was mixed with 20 μg biotin-labelled recombinant HaPrP in 1 mol/L GdnHCl, 50 mmol/L sodium citrate, pH 6.0, 1% N-laurylsarcosine, and 5 mmol/L cetyl pyridinium chloride, total volume of each reaction not exceeding 200 μL. The reactions were kept at 37℃ for 4 days with gentle stirring. After incubation, each reaction was divided into two aliquots, one being immediately employed into the digestion with 20 μg/mL of PK at 37℃ for 1 h and the other being incubated at the same condition but without PK. Both fractions were separated in 15% SDS-PAGE and electronically transferred onto nitrocellulose membrane. The PK-resistant signals were developed with HRP conjugated streptavidin and visualized in DAB substrate.

RESULTS

Expression of Full-length Hamster PrP in E. coli and Biotin Labelling

A 209aa hamster PrP gene was released from plasmid pT-HaPrP and inserted into plasmid pQE30 and transformed into *E. coli* M15. Using affinity chromatography of Ni-NTA agarose, HIS-fusion proteins were isolated from the *E. coli* cell lysate. SDS-PAGE revealed that the purified protein mobilized at approximately 26 kDa (Fig. 1A). It was estimated that approximately 2 mg proteins were purified from 100 mL culture. Western blot analysis, using an anti-PrP monoclonal antibody 3F4, showed a specific reactive band at the expected mobility position (Fig. 1B). The harvested HaPrP was subsequently employed into biotin labelling procedure. To address the biotin-labelling sensitivity, various amounts of the labelled HaPrP from 50 to 1000 ng were input into SDS-PAGE. After being incubated with HRP conjugated streptavidin and developed in DAB substrate, a single band was

observed in each preparation, with remarkable dose-dependant relationship (Fig. 2). At least 50 ng labelled protein could be easily detected in this reaction system.

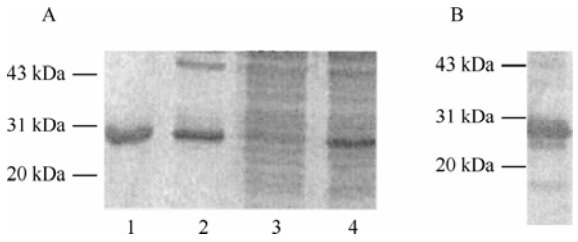

FIG. 1. Purification (A) and Western blot analysis (B) of HaPrP in *E. coli*. Lane 1: purified HaPrP23-231; lane 2: pellet after sonication; lane 3: supernatant after sonication; lane 4: lysate of *E. coli* transformed with pQE-HaPrP23-231 after inducement.

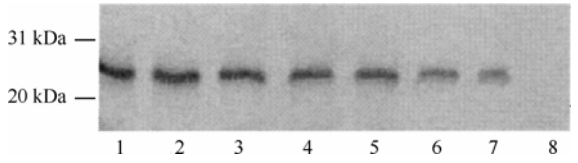

FIG. 2. Analyses of biotin labelled HaPrP by incubating with horseradish peroxidase conjugated streptavidin and developing in DAB substrate. Lanes 1 to 7: biotin labelled HaPrP with the concentrations of 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05 μg, respectively; lane 8: unlabelled HaPrP.

Extraction of PrP^{Sc} from Hamsters' Brains Infected with Scrapie Strain 263K

To prepare PrP^{Sc} for conversion test, the brain tissues from the infected hamsters were homogenized and the PrP^{Sc} proteins were partially purified. PK resistant proteins that mobilized around 21 to 30 kDa were identified by Western blot assay, in which di-glycosylation pattern of PrP^{Sc} was clearly predominant (Fig. 3). The concentration of crude PrP^{Sc} was estimated to be about 260 μg PrP^{Sc} per gram of the pool of infected hamsters' brains.

Biotin-labelled HaPrP Converted into PK-Resistant Isoform in Cell Free Conversion

To see whether the biotin-labelled and prokaryotic expressed recombinant PrP could be used in the cell free conversion, biotin-labelled HaPrP and hamster PrP^{Sc} were incubated in the conversion buffer for 4 days and the presence of PrP signals in all preparations was evaluated by Western blot. The PrP signals were detectable in the preparations of biotin-labelled HaPrP mixed with (Fig. 4, lane 1) or without (Fig. 4, lane 3) PrP^{Sc} prior to PK digestion, indicating that the biotin-labelled HaPrP was stable in the conversion buffer. After treatment with PK, the HaPrP signal disappeared in the preparation without PrP^{Sc}, suggesting that the the input HaPrP was PK-sensitive (Fig. 4, lane 2). A remarkable PK-resistant band was observed in the preparation of biotin-labelled HaPrP incubated with PrP^{Sc}, which mobilized roughly at 20 kDa (Fig. 4, lane 6), suggesting that the biotin-labelled HaPrP could be converted into PK-resistant isoform by the PrP^{Sc} preparation from scrapie infected hamsters. In line with the previous results obtained from the conversion assay with radioactive ^{35}S-meth; onine[10], incubation for 72 and 96 h showed no difference (data not shown).

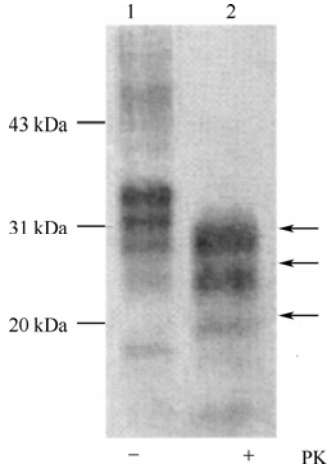

FIG. 3. Western blot analysis of PrP proteins isolated from the hamsters' brains infected with scrapie strain 263K. Lane 1: without PK; lane 2: with PK. Arrows indicate the protease resistant molecules (PrP^{res}).

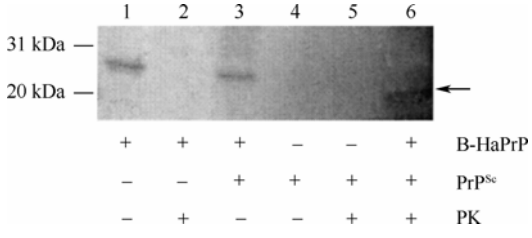

FIG. 4. Conversion of PK-sensitive biotin labelled HaPrP to PK-resistant isoform in cell free system. Lane 1: biotin labelled HaPrP only; lane 2: biotin labelled HaPrP treated with PK (20 μg/mL); lane 3: biotin labelled HaPrP with PrP^{Sc}, without treatment of PK; lane 4: PrP^{Sc} only; lane 5: PrP^{Sc} treated with 20 μg/mL PK; lane 6: biotin labelled HaPrP with PrP^{Sc} and treated with PK. Arrows indicate the newly formed protease resistant molecules (PrP^{res}).

DISCUSSION

In this study we described a method using biotin-labelled *E. coli*-expressed hamster PrP protein (HaPrP) in cell free conversion system, instead of conventionally used ^{35}S-labelled mammalian or insect cells-expressed PrP protein. The PK-sensitive HaPrP could be efficiently converted into PK-resistant one after being incubated with homogeneous PrP^{Sc} in a partially denaturing situation. Kocisko and his coworkers[4] have demonstrated that the glycosylation or GPI anchor of PrP protein is not indispensable for the PK resistant conversion in cell free system. Our previous report and other studies have also confirmed that the PrP proteins expressed in insect cells, possibly with incomplete glycosylation as well as incomplete tertiary structural conformation, are comparably efficient in cell free conversion as those expressed in mammalian cells[9-10]. Recently, Kirby has described a cell free conversion model using bacterial recombinant PrP[15]. Moreover, it has been confirmed again that HIS-tag in N-terminus of recombinant PrP has little effect on the conversion of PK-sensitive PrP to PK-resistant one *in vitro*.

Biotin, a small molecule substance of vitamin H, is widely used in antigen-antibody detection. In contrast with isotope, biotin can benefit experimenters and environments. No more considerations should be taken regarding the contamination and the half-life of biotin. According to our results, biotin seems to be very stable during the long-term incubation. Since biotin labelling procedure is carried out after purification of recombinant PrP, it allows to prepare a large quantity of labelled PrP protein in one preparation. Meanwhile, it is easy to control the labelling quality. All these confirm the advantage and perspective of using biotin labelled protein in cell free conversion assay instead of radiolabelled protein.

The PK resistant products in cell free conversion reactions have not yet been shown to be infectious. The major technical difficulties are the limited yields compared with PrP^{Sc}[16-17]. Many factors influence the conversion rates, e.g. input amounts of PrP^{Sc} and PrP^{C}, different strains of PrP^{Sc}, incubation conditions and chaperones[6,18-19]. A “cyclic amplification” procedure might help to achieve this goal[20]. Further optimization of the protocol with biotin labelling or other non-radioactive agents in the cell free conversion assay for PrP will provide a reliable, easily handling and environment-friendly methodology for prion and TSE studies.

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