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In vitro assay for fragmentation of amyloid fibers of yeast prion protein

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Abstract

In prion propagation, fragmentation of amyloid fibers, as well as conformational conversion of prion protein, is critical: the latter increases the net amount of abnormal prion proteins and the former multiplies number of seeds. We present here a method for in vitro measurement of fragmentation of amyloid fibers of yeast Sup35 prion protein. In this method, amyloid fibers are tethered to the surface of magnetic beads. Fragmentation of the fibers results in release of fiber fragments into the medium, which are then quantified by immunoblotting. This method is versatile for other amyloid fibers.

Keywords: Amyloid; Prion; Sup35; Fragmentation

1. Introduction

Prion propagation is primarily characterized by autocatalytic conformational conversion of prion protein from normal to abnormal form [1]. In addition to mammalian neurodegenerative diseases, prion phenomena have been seen in various organisms [2–5]. In yeast, prions are seen as non-Mendelian genetic elements [2], such as nonsense suppressor [PSI^+] [6], caused by conformational conversion of translation termination factor Sup35p [7–9]. In many cases, amyloid fibers consisting of protein molecules in abnormal conformation are generated. They are the ordered aggregates with cross β-sheet or β-helix structure [10–13]. At least for yeast prions, it does not seem that abnormal monomers by themselves assemble into amyloid fiber. Rather, the growing end of the fiber acts as a template for the newly bound normal monomer to convert into the abnormal one [14–17]. This contention is consistent with the observation that there are conformational variations among prion fibers, which are inherited over generations accompanying different sub-strains in prion phenotype. Fibers should be cleaved to generate smaller daughter fragments for efficient propagation and inheritance [18–21], because if the number of the fibers is not changed, the fibers would be diluted away after several generations. Studies both in vivo and in vitro have revealed that certain cellular factor(s) such as Hsp104 causes fragmentation of amyloid fibers of yeast prions [19–23]. To study fragmentation of yeast prion Sup35p fibers, we developed an in vitro assay method using amyloid fibers tethered to magnetic beads at one end. When the fiber is cleaved, the daughter fragment containing the non-tethered end will be released to the medium. This method provides sensitive detection of fragmentation, because a single cleavage event on each fiber can typically result in releasing half of the fiber mass. As mentioned, it is known that various sub-strains of [PSI^+] are generated by heterogeneous Sup35p fibers with conformational variations [24]. Although it is suggested that the differences in sub-strains relate to variations in the conversion efficiencies [25], the relationship between sub-strains and fragmentation efficiencies is still unclear. Sensitive measurement of fragmentation is required to know whether certain amyloid is cleavable or not, in other words, heritable or not. It is important because the definitive feature of prions that distinguishes them from other

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amyloid is infectivity, which correlates with inheritance or propagation. The method used here does not require any special equipment and may be applicable to other amyloidogenic proteins.

2. Overview of the method

Among numbers of amyloidogenic proteins, study of yeast prion protein Sup35p has several advantages such as easy fiber formation, powerful yeast genetics and handling safety. The N-terminal 253-amino acid fragment of Sup35 and its derivatives are widely used [7,11,26]. Purified Sup35(1–253) can spontaneously polymerize to form amyloid fibers after a short lag phase that is required for nucleation. The fibers are made from epitope-tagged Sup35(1–253) and tethered to magnetic beads by biotin–streptavidin conjugation. Bead-tethered fibers are incubated with yeast lysate (or components to be examined), and beads were magnetically removed. If the fibers are cleaved, fiber fragments should be released into the medium and detected by immunoblotting (Fig. 1).

3. Sample preparation

3.1. Proteins

We use here two kinds of Sup35(1–253)s that have either a His_8-Cys tag (Sup35NM) or a His_8-Cys-HA_2 tag (Sup35NMHA) at the C-terminus. The introduced cysteine residue of Sup35NMHA is used for fluorescent labeling to monitor sample preparation. It can be omitted in case fluorescent labeling is not required. Sup35NM and Sup35NMHA are expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL (Stratagene) by using the pET system (Novagen), and purified with a Ni–NTA column under denaturing conditions and with a reverse-phase column [14]. Purified protein is dried and stored at −20 °C.

3.2. Protein labeling

Dried Sup35NM and Sup35NMHA are solubilized at 100 μM in 8 M guanidine hydrochloride (pH 7.5) containing 200 μM TCEP. Sup35NM is reacted with 10-fold molar excess of biotin–$(PEAC)_5$–maleimide (PIERCE) or with *N*-ethylmaleimide (NEM). Sup35NMHA solution is reacted with Cy3 maleimide (Amersham) and residual free thiol should be eliminated by reactions with *N*-ethylmaleimide. Since Sup35(1–253) does not contain cysteine residues, only the introduced cysteines are reacted. The labeled proteins are adsorbed to a Ni–NTA column and free reagents are washed away. The eluted proteins are dialyzed against 0.05% trifluoroacetic acid and dried.

3.3. Fiber formation

Dried biotin-Sup35NM, NEM-Sup35NM and Cy3-Sup35NMHA are solubilized in 10 M urea, passed through a 100-kDa filter unit (Millipore), and the final concentrations are adjusted to 100 μM. Biotin-Sup35NM and NEM-Sup35NM solution are mixed at an appropriate ratio (biotin-/NEM-Sup35NM). This ratio should be optimized carefully, because congestion of biotins in the fiber tail may cause undesired coagulation of beads, which disturbs quantitative analysis. We have optimized it as 1:200. Fiber formation is started by diluting urea-denatured Cy3-Sup35NMHA 100-fold in Buffer A (20 mM Hepes[1], pH 7.5, 140 mM KCl, 15 mM NaCl, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT). The solution is incubated at room temperature for 2–3 days. A short-term agitation can be done at less than 10 rpm for less than 3 h at early phase. Vigorous or longer agitation is not recommended, because agitation produces many short fibers [17,26,27], which may cause low sensitivity (see Section 5). Urea-denatured biotin-/NEM-Sup35NM is then diluted 100-fold in Buffer A and immediately the solution is mixed with 5-fold excess volume of the Cy3-Sup35NMHA-fiber suspension. The mixture is incubated for another 3 days to introduce biotinylated tails on Cy3-Sup35NMHA fibers. To avoid fiber entanglement or clustering, the fiber suspension is stored after mixing with Triton X-100 to a final concentration of 0.05% until use.

3.4. Yeast lysate

We use here the lysate of *Saccharomyces cerevisiae* BY4741 strain. Cells are cultured until 4×10^7 cells/mL in YPD medium. Collected cells are finally suspended in PBS containing 5% glycerol, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail and broken with glass beads (425–600 μm, Sigma). Debris is removed by centrifugation followed by passing through a 0.4 μm filter. The lysate is adjusted to 4.0 mg protein/mL and stored at −20 °C.

4. Procedures to assay fragmentation

4.1. Reaction

The fiber solution is mixed with a suspension of streptavidin-magnetic beads (streptavidin-paramagnetic particles, Roche) at 10:1 (v/v) and incubated with gentle agitation for 3 h at 4 °C. If necessary, the magnetic beads can be pretreated by appropriate reagent to minimize non-specific adherence of proteins (we use PMB80 resin, which is kindly provided by Dr. Kazunari Akiyoshi, Tokyo Medical and Dental University). To remove free fibers, the beads are washed by magnetic separation and re-suspended in Buffer A containing 1% Triton

[1] *Abbreviations used:* Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; TCEP, tris-(2-carboxyethyl)phosphine.