Video Article

Use of Two Dimensional Semi-denaturing Detergent Agarose Gel Electrophoresis to Confirm Size Heterogeneity of Amyloid or Amyloid-like Fibers

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Abstract

Amyloid or amyloid-like fibers have been associated with many human diseases, and are now being discovered to be important for many signaling pathways. The ability to readily detect the formation of these fibers under various experimental conditions is essential for understanding their potential function. Many methods have been used to detect the fibers, but not without some drawbacks. For example, electron microscopy (EM), or staining with Congo Red or Thioflavin T often requires purification of the fibers. On the other hand, semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) allows detection of the SDS-resistant amyloid-like fibers in the cell extracts without purification. In addition, it allows the comparison of the size difference of the fibers. More importantly, it can be used to identify specific proteins within the fibers by Western blotting. It is less time consuming and more easily accessible to a wider number of labs. SDD-AGE results often show variable degree of heterogeneity. It raises the question whether part of the heterogeneity results from the dissociation of the protein complex during the electrophoresis in the presence of SDS. For this reason, we have employed a second dimension of SDD-AGE to determine if the size heterogeneity seen in SDD-AGE is truly a result of fiber heterogeneity *in vivo* and not a result of either degradation or dissociation of some of the proteins during electrophoresis. This method allows fast, qualitative confirmation that the amyloid or amyloid-like fibers are not partially dissociating during the SDD-AGE process.

Video Link

The video component of this article can be found at https://www.jove.com/video/57498/

Introduction

The formation of amyloid fibers due to protein misfolding has long been known to play a role in pathologic conditions such as Alzheimer's disease, Parkinson's disease, and Huntington's disease[1]. More recently, the formation of amyloid or amyloid-like fibers has been shown to be a part of signaling pathways in humans, including during anti-viral innate immune response[2] and necroptosis[3,4], and in lower organisms such as yeast[5,6]. Therefore, the ability to detect these fibers in the lab is important. Currently, there are three main ways to detect amyloid and amyloid-like fibers: the use of dyes, EM, and SDD-AGE.

The use of dyes, such as Congo Red or Thioflavin T, offers the advantage of being rapid and easily detectable using either microscopy or spectroscopy[7]. However, detection by microscopy, in the case of Congo Red, provides no specificity about which proteins comprise the fibers, or the size of the fibers. Similarly, the use of spectroscopy to detect Congo Red or Thioflavin T binding to protein complexes provides only a positive or negative result.

EM provides conclusive evidence of the presence of fibers and also quantitative information about the fiber length and diameter[8]. However, this method requires very stringent purification. Additionally, EM is a specialized technique which uses expensive equipment.

SDD-AGE has been used to detect SDS-resistant mega Dalton protein complexes including amyloid or amyloid-like fibers. It offers many advantages. First, it does not require the purification of the fibers and is easy to peform[9]. Second, it provides qualitative information about the size of the fibers, including relative size and amount of fiber heterogenicity. Lastly, because Western blotting can be performed after electrophoresis, it is easy to detect the presence of any protein for which there is an antibody, although it should be noted that because SDD-AGE is semi-denaturing, some epitopes may remain concealed which complicates detection by antibody.

Recently, receptor interacting protein kinase 1 (RIPK1) and 3 (RIPK3) have been reported to form amyloid fibers to serve as signaling platforms during necroptosis, a programmed form of necrosis[3]. While studying these fibers, our lab showed that another necroptosis-associated protein, mixed lineage kinase domain-like (MLKL), also formed amyloid-like fibers[4]. However, upon examination with SDD-AGE, the size of the MLKL

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fibers appeared distinct from the RIPK1 and RIPK3 fibers, which appeared identical to each other (**Figure 1**). This was unexpected because it is well-known that MLKL binds to RIPK1/RIPK3 to form the necroptosis signaling complex called the necrosome[10].

There are at least two explanations. First, two totally distinct amyloid-like fibers may form during necroptosis, one containing RIPK1/RIPK3 and the other containing MLKL. Second, only one type of amyloid-like fibers containing RIPK1/RIPK3/MLKL may be formed during necroptosis, but the association of MLKL with the other proteins is weak enough that it dissociates during SDD-AGE.

To address this, we propose performing a two-dimensional (2D) SDD-AGE. SDD-AGE-stable amyloid or amyloid-like fibers will have the same migration pattern during the first and second dimension electrophoresis. This will be detectable after transferring the proteins to a membrane and carrying out a Western blot. Stable fibers will exhibit a sharp diagonal pattern. Any deviation from this would suggest that the fibers undergo changes due to the SDS-electrophoresis.

Protocol

1. Prepare Samples

1. Culture 2 x 10^6 amyloid producing HT-29 colon cancer cells in a 10-cm tissue culture dish in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and penicillin-streptomycin. Culture cells overnight in a 37 °C incubator with 5% CO_2.
    1. After the cells grow to 80% confluency, wash the cells with 10 mL of phosphate-buffered saline (PBS). Add 3 mL of Trypsin solution and incubate at 37 °C for 3 min.
    2. After the cells are totally dissociated from the dish, add 10 mL of culture medium and transfer the cells with a 10-mL pipette to a 15-mL conical tube. Centrifuge the cells at 1,000 x g for 3 min at room temperature. Aspirate the medium, resuspend the cells in 5 mL of culture medium, and count the cells using a cell counter. Plate 2 x 10^6 cells in each of two 10-cm dishes.
    3. Allow the cells to adhere and recover overnight in a 37 °C incubator with 5% CO_2. Apply treatment to one dish to induce the formation of amyloids with 20 ng/mL Tumor Necrosis Factor-Alpha (TNF-α), 100 nM Smac-mimetic, and 20 µM pan-caspase inhibitor Z-VAD-FMK[11]. The combination is abbreviated as TSZ. Treat the other dish with vehicle as a control.

2. After the appropriate length of time, usually 6 h, harvest the cell lysate.
    1. Scrape the cells off the plate with a plastic scraper and use a 10-mL pipette to transfer into a 15-mL conical tube. Centrifuge the cells at 1,000 x g for 3 min at 4 °C.
    2. Wash the cells 2 times by resuspending in 10 mL of ice cold PBS and centrifuging at 1,000 x g for 3 min at 4 °C. Aspirate the PBS solution.
    Note: The process can be paused here by freezing the cell pellet in liquid nitrogen and storing at -80 °C for up to 1 month.
    3. Transfer the cell pellet to a 1.5-mL microcentrifuge tube and incubate in 0.3 mL of lysis buffer for 30 min on ice. Centrifuge at 20,000 x g for 15 min at 4 °C. The supernatant is the whole cell lysate.
    Note: The process can be paused here by storing the sample at -20 °C for up to several months.
    4. Measure the protein concentration by a Bradford assay according to manufacturer's protocol. Add 4x SDD-AGE loading buffer to prepare 20 µL of 3 µg/µL sample and incubate at room temperature for 10 min.

2. Prepare and Run Gels

1. Add 2 g of agarose to 200 mL of 1x Tris-acetate buffer (TAE) in a glass beaker and heat in a microwave to melt the agarose. Add 1 mL of 20% SDS for a final concentration of 0.1% SDS. Carefully swirl to mix. Take care not to generate bubbles after the SDS addition.
2. Pour the agarose solution into a 15 cm x 14 cm gel slab. Use a 1-mL pipette to eliminate any bubbles. Place one 20-well comb at the top.
3. First dimension: Pipette 60 µg of whole cell lysate in the far-right lane. Run the gel at 60 V for about 4 h (until the dye front is about ¾ through the gel) using the TAE containing 0.1% SDS as the running buffer.
4. Second dimension: Carefully rotate the gel 90° counter-clockwise (**Figure 2A**). Run the gel at 60 V for about 4 h.
Note: The general running condition is 4 V/cm gel length. It is important that the running conditions are exactly the same for the first and second dimensions.

3. Transfer

1. Use the capillary transfer method to transfer the proteins to a polyvinylidene difluoride (PVDF) membrane (**Figure 2B**).
    1. Add 500 mL of transfer buffer to an approximately 20 cm x 20 cm container. Make a 5-cm high stack of paper towels next to the container. The surface area of the top of the stack must be greater than the gel dimensions.
    2. Soak a 14 cm x 15 cm filter paper in transfer buffer and place on top of the paper towel stack. Take care to place on the paper with no wrinkles or bubbles. Repeat with another piece of filter paper.
    3. Activate a 14 cm x 15 cm PVDF membrane in methanol for 30 s then layer it on the filter paper taking care to use a roller to remove all bubbles. Rinse the gel off with transfer buffer and layer it on top of the membrane, again rolling out any bubbles.
    4. Cover the edge of the paper towels closest to the container of transfer buffer with plastic wrap. It is important that the paper bridge constructed in the next step does not come into direct contact with the paper towel stack.
    5. Soak a piece of 15 cm x 35 cm filter paper in transfer buffer and place it so one end covers the top of the gel and the other end is in the container of transfer buffer. Repeat with another bridge.
    6. Cover the container with plastic wrap and leave overnight at room temperature.

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4. Western Blotting Detection

1. Rinse the membrane with 50 mL of PBS containing 0.05% Tween-20 (PBST) in a 20 cm × 20 cm container. Add 20 mL of 5% milk in PBST. Rock at room temperature for 30 min to block.
2. Pipette 10 µL of rabbit anti-MLKL antibody into 20 mL of 5% milk in PBST and add to the container. Incubate on rocker overnight at 4 °C.
3. Wash the membrane in 20 mL of PBST for 5 min. Repeat 5 times.
4. Pipette 4 µL of anti-rabbit-HRP to 20 mL of 5% milk in PBST and add to the container. Incubate on rocker at room temperature for 2 h.
5. Wash the membrane in 20 mL of PBST for 5 min. Repeat 5 times.
6. Add enhanced chemiluminescence substrate (ECL) and expose to X-ray films according to the manufacturer's protocol.

Representative Results

After necroptosis induction, RIPK1 and RIPK3 showed almost identical amyloid-like patterns (lanes 2 and 4, **Figure 1**). However, MLKL fibers were more heterogeneous and seemed to be smaller than RIPK1/RIPK3 fibers (lane 6, **Figure 1**). That prompted us to develop the 2D SDD-AGE method to address the possibility whether MLKL forms RIPK1/RIPK3-independent fibers or MLKL simply dissociates from the large RIPK1/RIPK3/MLKL fibers during SDD-AGE.

A general schematic of the electrophoresis and transfer procedure is shown in **Figure 2**. During the first dimension SDD-AGE, amyloid or amyloid-like fibers will exhibit a characteristic smear, as seen in **Figure 3A**. Upon running the second dimension SDD-AGE, there are two possible outcomes. In the case of no degradation or dissociation during the gel running process, the fibers will migrate identically during the second run as they did in the first run. In this case, a Western blot will exhibit a diagonal pattern on the membrane at a 45° angle as seen in **Figure 3B**. If the fibers dissociate during the SDD-AGE process, the smaller fibers will migrate faster during the second electrophoresis. In this case, a Western blot will exhibit vertical streaking below the diagonal line, as shown in **Figure 3C**.

In the case of the MLKL fibers seen during necroptosis, they show the characteristic smear during the first dimension SDD-AGE (**Figure 4A**). When those same fibers were subjected to 2D SDD-AGE, they exhibited a sharp diagonal line with no vertical streaking (**Figure 4B**). With this evidence, it was concluded that MLKL was not dissociating from the fibers during the SDD-AGE process and that the MLKL-containing fibers seen in **Figure 1** were indeed distinct from the RIPK1/RIPK3 fibers. Furthermore, when RIPK3 was immuno-depleted from the cell lysates, the MLKL fibers remained intact[4], confirming that MLKL fibers and RIPK1/RIPK3 fibers are indeed distinct entities.

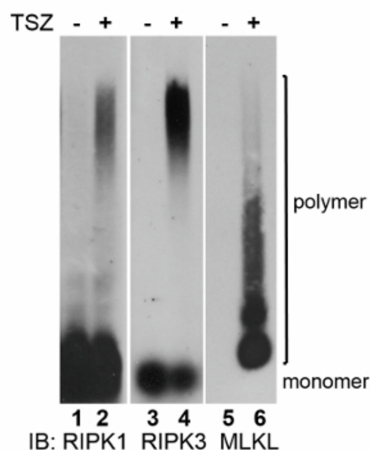

Figure 1. Examination of amyloid-like fibers during necroptosis. Whole cell lysates were harvested from cells undergoing TNF-induced necroptosis and subjected to SDD-AGE. Western blots were performed for RIPK1, RIPK3, and MLKL. The MLKL-containing fibers exhibited a distinct migration pattern from the RIPK1/RIPK3-containing fibers. The MLKL monomer is barely visible under this condition. Please click here to view a larger version of this figure.

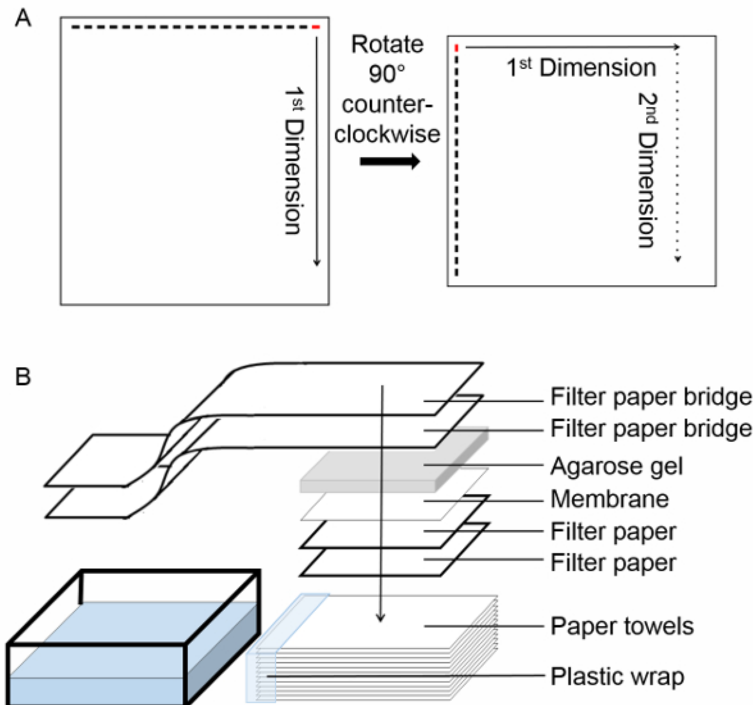

Figure 2. Experimental protocol. (**A**) Schematic of the two-dimensional semi-denaturing detergent agarose gel electrophoresis (2D SDD-AGE). Begin by loading the sample in the right most lane, labeled in red. After termination of the first dimension run, rotate the gel 90° counter-clockwise. Run the second dimension electrophoresis. Solid arrow indicates the direction of the first dimension run, dotted arrow indicates the direction of the second dimension run. (**B**) Schema of transfer by capillary action. Please click here to view a larger version of this figure.

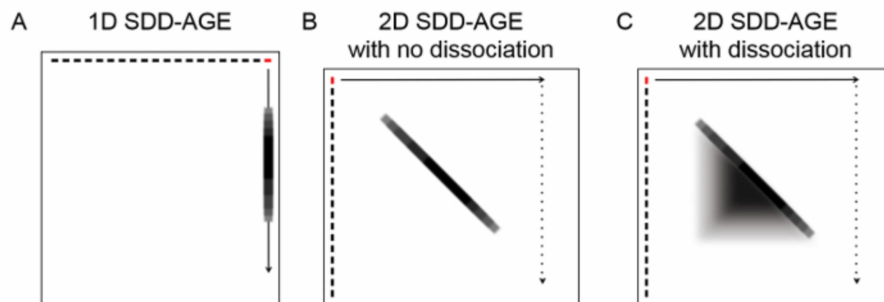

Figure 3. Possible results. (**A**) The expected migration pattern of amyloid or amyloid-like fibers after the first dimension of SDD-AGE. Solid arrow indicates the direction of migration. (**B**) The expected migration pattern of the SDD-AGE-stable (no degradation or dissociation) amyloid or amyloid-like fibers after 2D SDD-AGE. Solid arrow indicates the direction of the first dimension SDD-AGE. Dotted arrow indicates the direction of the second dimension SDD-AGE. (**C**) The expected migration pattern of the non-SDD-AGE-stable amyloid or amyloid-like fibers. Solid arrow indicates the direction of the first dimension SDD-AGE. Dotted arrow indicates the direction of the second dimension SDD-AGE. Please click here to view a larger version of this figure.

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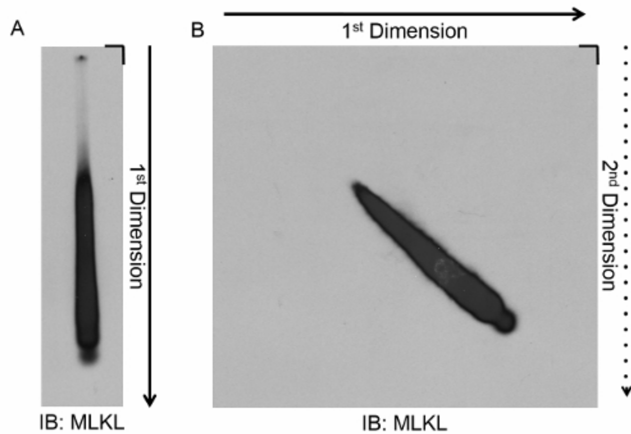

Figure 4. **Migration of MLKL-containing fibers during 1D and 2D SDD-AGE.** (**A**) Whole cell lysates were harvested from cells stimulated with TSZ to undergo necroptosis. The lysates were subjected to SDD-AGE, and a Western blot was performed for MLKL. A characteristic smear was observed. (**B**) Whole cell lysates were harvested from cells stimulated with TSZ to undergo necroptosis. The lysates were subjected to 2D SDD-AGE and a Western blot was performed for MLKL. A sharp line at a 45° angle was observed, indicating that the fibers do not undergo dissociation during SDD-AGE. Please click here to view a larger version of this figure.

Discussion

The most critical aspect of the 2D SDD-AGE is that the electrophoresis conditions are the same for the first and second dimension. The ability to detect a sharp diagonal line at 45° (indicating that no dissociation or degradation has occurred) depends on the fibers migrating in an identical manner during both electrophoreses. Using different conditions, for example changing the voltage or the length of run time, will obscure these results. Also, as is the case for traditional 1D SDD-AGE, boiling the sample should be avoided, as this will disrupt amyloid and amyloid-like fibers. As is the case for all transfers, care should be taken to avoid bubbles between the layers of filter paper, membrane, and gel. Lastly, during the transfer step, it is critical that the bridge does not droop and directly contact the paper towel stack. A reliable way to prevent this is to use a small piece of plastic wrap to cover the edge of the paper towels.

The running conditions provided in the protocol (60 V for 4 h) may be adjusted. For example, in this protocol, the agarose gel was 14 cm x 15 cm. If a smaller gel is used, voltage (4 V/cm gel length) and run time should be adjusted proportionally. Two samples may be analyzed at once by using two combs when preparing the gel. The samples should be loaded in the right most well for each comb. In this case, the run time should again be shortened so that the top sample does not run into the bottom half of the gel. If the time is adjusted for the one dimension of the gel, it is critical that the other dimension is adjusted to match.

In contrast to SDS-PAGE, both 1D and 2D SDD-AGE are semi-denaturing. Because the fibers remain intact, some epitopes might remain inaccessible. This may limit detection by some antibodies. 2D SDD-AGE is limited in its ability to determine the exact size of amyloid or amyloid-like fibers. However, a relative size comparison may be made.

All protein complexes are subject to dissociation or degradation during SDD-AGE, and so the qualitative results may be difficult to interpret. The use of 2D SDD-AGE permits confirmation that size heterogeneity seen during SDD-AGE is not due to SDS-dependent dissociation during electrophoresis. 2D SDD-AGE is appropriate to use in any context where traditional 1D SDD-AGE is used. 2D SDD-AGE is especially useful when size heterogeneity is observed in 1D SDD-AGE.

Disclosures

The authors declare no conflict of interest.

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