Chapter 21

Analysis of Protein Oligomeric Species by Sucrose Gradients

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Abstract

Protein misfolding, aggregation, and accumulation are a common hallmark in various neurodegenerative diseases. Invariably, the process of protein aggregation is associated with both a loss of the normal biological function of the protein and a gain of toxic function that ultimately leads to cell death. The precise origin of protein cytotoxicity is presently unclear but the predominant theory posits that smaller oligomeric species are more toxic than larger aggregated forms. While there is still no consensus on this subject, this is a central question that needs to be addressed in order to enable the design of novel and more effective therapeutic strategies. Accordingly, the development and utilization of approaches that allow the biochemical characterization of the formed oligomeric species in a given cellular or animal model will enable the correlation with cytotoxicity and other parameters of interest.

Here, we provide a detailed description of a low-cost protocol for the analysis of protein oligomeric species from both yeast and mammalian cell lines models, based on their separation according to sedimentation velocity using high-speed centrifugation in sucrose gradients. This approach is an adaptation of existing protocols that enabled us to overcome existing technical issues and obtain reliable results that are instrumental for the characterization of the types of protein aggregates formed by different proteins of interest in the context of neurodegenerative disorders.

Key words Protein misfolding, Yeast model, Mammalian cell lines, Proteinopathies, Oligomers, Protein aggregates, Sucrose gradients, Velocity sedimentation, High-speed centrifugation, Neurodegenerative diseases

1 Introduction

Proteinopathies are a group of diseases that share in common the misfolding, aggregation, and accumulation of specific proteins in different tissues, from peripheral nerves and organs, to the central nervous system. The aggregation process is thought to evolve through the formation of protein dimers, oligomers, protofibrils and, ultimately, amyloid fibrils organized in large aggregates [1]. Two consequences might derive from the aggregation process: a loss of the normal function of the protein involved, and a gain of

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toxic function of the misfolded and aggregated forms. However, the precise nature of the toxic forms is still controversial, with recent data suggesting oligomeric forms might be more toxic than larger, insoluble, aggregated species. The clarification of these questions is central for the understanding of the molecular basis of many disorders and for the development of effective therapeutic strategies. Thus, intense efforts have been dedicated to the clarification of this question, through the use of in vitro approaches combined with the use of cellular and animal models. Accordingly, the establishment of a correlation between aggregation and cytotoxicity induced by a given misfolded protein in the various model systems is crucial.

Yeast models of proteinopathies, such as Parkinson's disease (PD), have provided important contributions to the current knowledge on the molecular pathways associated with the toxicity of alpha-synuclein (aSyn), the protein that misfolds and aggregates in PD [2]. In particular, aSyn was found to be toxic and to form cytoplasmic inclusions in yeast cells [3]. However, the precise biochemical nature of the aSyn species formed in yeast was unclear. Here, we demonstrate the adaptation of a technique that allows the biochemical analysis of the aggregation state of aSyn. This approach can also be used to study other proteins of interest and is applicable for the study of protein extracts obtained from different cells and tissues. This protocol is based on density gradient sedimentation, where oligomeric species sediment through the gradient in separate zones based on their sedimentation rate.

Briefly, yeast cell lysates are generated by spheroplasting, in order to preserve the proteins in their physiological state, and then a defined protein amount is applied at the top of a discontinuous sucrose gradient (5–30% (w/v)). The various protein species are separated by ultracentrifugation, and fractions are collected from the top of the gradient. The protein is then precipitated, washed, solubilized, and resolved in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE), after which it is transferred electrophoretically to a nitrocellulose membrane. The protein of interest can then be visualized by immunoblotting with specific antibodies (Fig. 1).

Using this method we demonstrated that, in yeast cells, aSyn aggregation is promoted by coexpression with the polo-like kinase 2 PLK2 [4] and is inhibited by the chemical chaperone mannosylglycerate [5], by S129 phosphorylation [6], or by coexpression with Hsp31–34, the yeast DJ-1 orthologs [7].

2 Materials

All solutions are prepared using ultrapure water (prepared by purifying deionized water to attain a resistivity of 18 M Ω cm at 25 °C) and analytical grade reagents. Solutions are stored at room

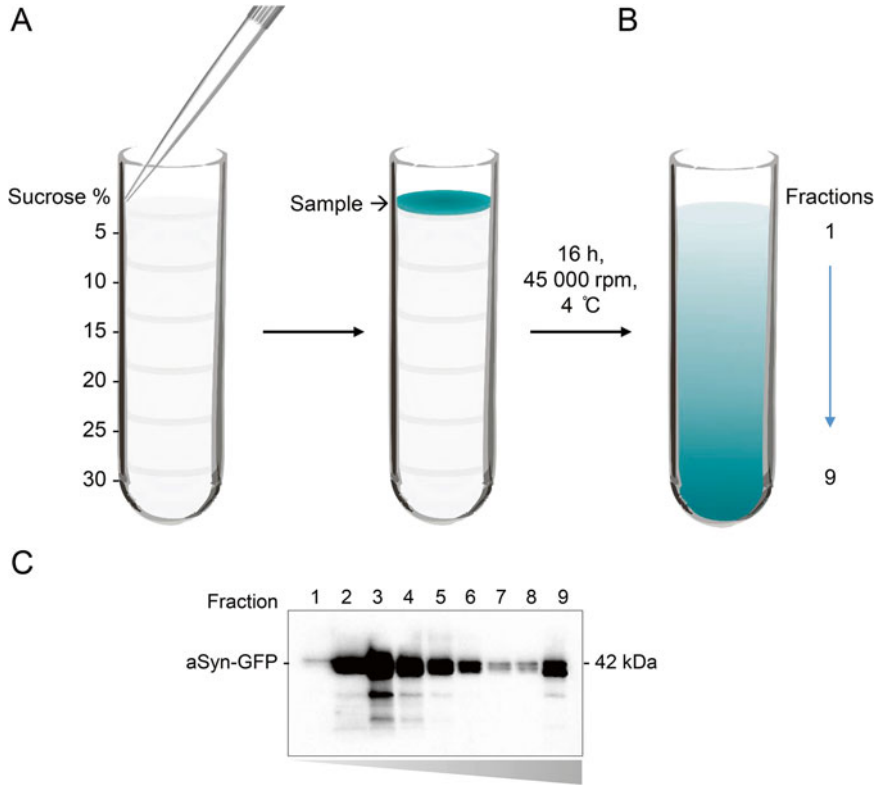

Fig. 1 Sucrose gradient preparation and immunoblotting. (**a**) The different sucrose solutions are carefully pipetted into the centrifuge tubes and the sample is loaded on top. (**b**) After centrifugation the components of the sample are separated based on their size and fractions 1–9 are collected. (**c**) The fractions, corresponding to different proteins sizes, are subjected to an SDS-PAGE and immunoblotting for the protein of interest

temperature unless indicated otherwise. Materials needed are: falcon and eppendorf centrifuges, ultracentrifuge, 30 and 95 °C incubator or bath, 2.5 mL syringes, 25G needles, 5 mL thinwall polypropylene centrifuge tubes, trichloroacetic acid (TCA), acetone, protease inhibitors.

2.1 Sucrose Gradient Solutions

1. Phosphate-buffered saline (PBS): 0.13 M NaCl, 2.7 M KCl, 12.5 M Na_2HPO_4, 1.76 M KH_2PO_4. Mix 80 g of NaCl, 2 g of KCl, 17.8 g of Na_2HPO_4, and 2.4 g of KH_2PO_4, in 800 mL of water. Adjust pH to 7.4. Complete the volume of the solution to 1 L with water. Before using, dilute 10 mL of PBS solution in 100 mL of water.
2. 80% (w/v) Sucrose: Weight 8 g of sucrose, adjust the volume to 100 mL of water and dissolve it well. Filter it using a 0.22 μm pore size filter (for aqueous solutions) and store at 4 °C to avoid contamination with microorganisms.

3. 1 M Tris: Weigh 121.1 g of Tris base and add water to a volume of 800 mL. Mix and adjust pH to 7.4 or 6.8 by adding concentrated HCl. Adjust the volume of the solution to 1 L with water and sterilize by autoclaving 15 min at 121 °C (*see* **Note 1**).
4. 1 M Magnesium chloride ($MgCl_2$): Dissolve 57.6 g of $MgCl_2$ in 400 mL of water. Adjust the volume to 500 mL with water, dispense into aliquots and sterilize by autoclaving 15 min at 121 °C (*see* **Note 2**).
5. 5000 U/mL Zymolyase 100T: Weight 1 g of zymolyase 100T and dissolve it in 20 mL of water or specific supplied buffer (*see* **Note 3**).
6. 5 M Sodium Chloride (NaCl): Dissolve 292.2 g of NaCl in 800 mL of water. Adjust the volume to 1 L with water, dispense into aliquots, and sterilize by autoclaving.
7. 350 mM Sodium dodecyl sulfate (SDS): Dissolve 100 g of SDS in 900 mL of water. Heat to 68 °C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 L with water and dispense into aliquots (*see* **Note 4**).
8. Protein sample buffer: 0.5 M Tris pH 6.8, 10% glycerol, 3.5 mM SDS, 6 mM bromophenol blue. Mix 25 mL of 1 M Tris pH 6.8, 5 mL of glycerol, 0.5 mL of 350 M SDS and 0.2 g of bromophenol blue. Complete the volume to 50 mL with water and aliquot. Store at −20 °C (*see* **Note 5**).
9. Spheroplasting solution: 20 mM Tris pH 7.4, 0.5 mM $MgCl_2$, 50 mM beta-mercaptoethanol, 1.2 M D-sorbitol, 50 U/mL zymolyase 100T. In a vial, add 5 mL of water, 200 µL of 1 M Tris pH 7.4, 5 µL of 1 M $MgCl_2$, 2.19 g of D-Sorbitol. Adjust the volume to 10 mL with water and mix. Store at −20 °C. Just before starting the experiment add 36 µL of beta-mercaptoethanol and 100 µL of zymolyase 100T at 5000 U/mL (*see* **Note 6**).
10. Lysis buffer: 20 mM Tris pH 7.4, 100 mM NaCl, 14 mM SDS, 0.2% Triton X-100. Prepare the lysis buffer by adding 5 mL of 1 M Tris pH 7.4, 5 mL of 5 M NaCl, 10 mL of 350 mM SDS and 0.5 mL of Triton X-100. Adjust the volume to 250 mL with water and mix very well. Aliquote and store at −20 °C (*see* **Note 7**).

3 Methods

All procedures are carried out at room temperature unless otherwise specified.

3.1 Preparation of Yeast Cells

1. Thaw the spheroplasting solution and lysis buffer, and keep them on ice.
2. Measure the OD_{600nm} of the yeast culture in a spectrophotometer.
3. Calculate the volume of culture corresponding to 5 OD_{600nm}/mL.
4. Place the determined volume in a vial and centrifuge at 800 × *g* for 4 min.
5. Discard the supernatant.
6. Resuspend cells in 1 mL of sterile water and transfer to a 1.5 mL eppendorf.
7. Pellet the cells by centrifuging at 800 × *g* for 3 min and remove the supernatant.
8. Resuspend cells in 1 mL of spheroplasting solution and incubate at 30 °C for 30 min (*see* **Note 8**).
9. Centrifuge at 800 × *g* for 5 min.
10. Remove the supernatant.
11. Add protease inhibitors to the lysis buffer, mix by vortexing. Add 1 tablet of cOmplete Mini, EDTA-free (Roche, Mannheim, Germany) to 10 mL of lysis buffer. It can be stored at –20 °C for 2 months.
12. Resuspend cells in 500 µL of lysis buffer for velocity with inhibitors and keep them 20 min on ice.
13. Break the cells by forcing the solution to pass through a 25G needle syringe 6 times. Use a different syringe and needle for each solution (*see* **Note 9**).
14. Proceed with protein quantification by the method of your preference.
15. Proceed to Subheading 3.3.

3.2 Preparation of Mammalian Cells

1. Grow cells as usual in six well plates.
2. When cells are 90% confluent place the plates on ice.
3. Remove the medium and gently wash cells three times with phosphate-buffered saline (PBS).
4. Add 100 µL of lysis buffer with inhibitors and keep them 20 min on ice.
5. Collect protein by scrapping the wells and pipette it to a 1.5 mL eppendorf.
6. Break the cells by forcing the solution to pass through a 25G needle syringe 6 times. Use a different syringe and needle for each solution (*see* **Note 9**).
7. Proceed with protein quantification by the method of your preference.
8. Proceed to Subheading 3.3.

3.3 Sucrose Gradients

1. While protein is being quantified prepare the following sucrose solutions:

#	Sucrose %	Sucrose 80 % (mL)	Lysis buffer (mL)
1	30	2.2	3.8
2	25	1.9	4.1
3	20	1.5	4.5
4	15	1.1	4.9
5	10	0.75	5.3
6	5	0.375	5.6

2. Approximately 15 min before the protein quantification protocol finishes, pipette 700 μL of each sucrose solution, from 1 to 6, in a 5 mL thinwall polypropylene centrifuge tube (from Beckman) (Fig. 1a). Do this gently and with the pipette tip close to the tube wall. You should be able to see an interface between sucrose solutions (*see* **Note 10**).
3. On top of each centrifuge tube, containing the gradient, place the desired total amount of protein: we use 1 mg (*see* **Note 11**).
4. Place the centrifuge tubes in the appropriate ultracentrifuge rotor tubes.
5. Carefully weight and calibrate the rotor tubes by weighting the opposing pairs and adjusting the weight with sucrose solution 6 (5 % sucrose in lysis buffer, in Subheading 3.3, **step 1**).
6. Carefully place the rotor tubes in the rotor and ultracentrifuge at 246,000 × *g*, 4 °C during 16 h in a swinging bucket rotor (SW-55Ti rotor, Beckman Instruments, Co., Palo Alto, CA), Beckman XL-90 S/N ultracentrifuge (*see* **Note 9**).
7. Immediately collect volumes of 500 μL from the gradients to 1.5 mL eppendorfs (Fig. 1b). Number them fraction 1–9 and keep them on ice (*see* **Note 12**).
8. Add 125 μL (40 M) of trichloroacetic acid (TCA) to each fraction and place for 4 h at 4 °C. TCA is corrosive and should be handled in the fume hood.
9. Centrifuge at 16,000 × *g* for 5 min.
10. Add 800 μL of acetone to each fraction, mix, and centrifuge at 16,000 × *g* for 5 min. Repeat this step two more times, keeping the samples on ice.
11. Place 1–2 min at 95 °C, but do not dry the samples excessively.
12. Add 20 μL of PSB and resuspend very well (*see* **Note 13**).

13. At this point the fractions can be stored at −20 °C until further used.
14. Place the samples for 10 min at 95 °C.
15. Run samples in an SDS-PAGE and do immunoblotting for the protein of your interest (Fig. 1c) (*see* **Notes 14** and **15**).

4 Notes

1. Having water at the bottom of the cylinder helps to dissolve Tris. Use a magnetic stir bar if necessary. Tris can be dissolved faster using warmed water to about 37 °C. Allow the solution to cool down to room temperature before making the final adjustments to the pH. After adjusting the volume of the solution to 1 L with water, dispense into aliquots, and sterilize by autoclaving 15 min at 121 °C. If the 1 M solution of Tris has a yellow color discard it and obtain better quality Tris. The pH of Tris solutions is temperature-dependent, and decreases approximately 0.03 pH units for each 1 °C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5, 25, and 37 °C, respectively. Concentrated HCl (12 N) can be used at first to narrow the gap from the starting pH to the required pH. From then on it would be better to use a series of HCl (e.g., 6 and 1 N) to avoid a sudden drop in pH below the required pH.
2. $MgCl_2$ is extremely hygroscopic. Buy small bottles (e.g., 100 g) and do not store open bottles for long periods of time.
3. The solubility of Zymolyase 100T is very low, it may not be completely dissolved in buffers, you may use as suspension. It is stable for over 1 year at −20 °C or many years below −70 °C. However, about 70% of the lytic activity is lost when stored at 30 °C for 3 months.
4. Wear mask when weighing SDS. Wipe down the weighing area and balance after use, because the fine crystals of SDS disperse easily. There is no need to sterilize 10% SDS.
5. Care should be taken to add SDS solution last, since it makes bubbles. SDS precipitates at 4 °C. Thus, the solutions with SDS needs to be warmed prior to use, if needed vortex the solutions.
6. Work with beta-mercaptoethanol in the fume hood, since it is very toxic and has a pungent smell.
7. To obtain the correct volume of Triton X-100 pipette it slowly, because it is very tick.
8. This step could require optimization. Spheroplasting efficiency depends on yeast strain, growth stage, and culture conditions.

Spheroplasting efficiency could be evaluated by microscopy or by absorbance at OD_{600nm}. Under phase-contrast light microscopy, spheroplasts will display a round morphology while intact yeast cells will present an ovoid shape. For OD_{600nm} evaluation, dilute 10 μL of spheroplasting solution into 1 mL of water. Total spheroplasting conversion will result in a reduction of 5–10% of the initial OD_{600nm} [8].

9. The cell lysates should be maintained on ice during protein quantification and should be used in the sucrose gradient immediately without being frozen, to avoid the risk of alteration of the oligomeric forms of the protein of interest.
10. The more concentrated sucrose solution, solution 1 in Subheading 3.3, **step 1**, will stay in the bottom and the less concentrated, solution 6 in Subheading 3.3, **step 1**, on top. Tubes should be placed securely in a customized support to protect the gradients from disturbance during preparation. Add each one of the solutions, from 1 to 6, very slowly, placing the tip against the internal wall of the centrifuge tube. It is very important to avoid the formation of bubbles. Do not take more that 20–30 min between preparing the tubes and starting the centrifugation. From this step onwards carefully transport the gradient centrifuge tubes, to avoid mixing the sucrose solutions.
11. The volume of different samples should be adjusted to the same final volume with lysis buffer. Volume to be applied to the gradient should not be more than 300 μL.
12. Collect the fraction immediately to avoid mixing the sucrose fractions. Fraction 1 will be the less concentrated and fraction 9 the most concentrated. The heavier proteins or aggregates will be found at the bottom fractions and the lighter at the top fractions (Fig. 1).
13. The more concentrated fractions are harder to dissolve and can acquire a yellow coloration, due to the accumulation of higher proteins/aggregates and concomitant change in pH. We found useful to do cycles of adding protein sample buffer and vortexing, but care should be taken to keep the volume of sample within the capacity of the SDS-PAGE wells. Also do a spin-down before loading the samples into the wells.
14. When comparing treatments/conditions make sure that the differences obtained in the sucrose gradients are not due to different protein expression levels. For this, load the same concentration of total protein in an SDS-PAGE and do immunoblotting for the protein of interest and for a loading control (e.g. beta-actin, GAPDH). The sucrose gradient bands can be quantified by doing densitometry of the bands in each fraction and normalizing in comparison to the sum of all bands. The results are represented as percentage of your protein in each fraction.

15. For protocol optimization native molecular mass markers, of known size and sedimentation coefficients, to determine the molecular weights corresponding to each sucrose gradient fraction, might be useful [9]. Calibration proteins for gel filtration chromatography could be used as marker proteins. e.g. gel filtration calibration kit from GE Healthcare, Boehringer Mannheim, or Sigma. Briefly, a mixture containing sufficient quantity of each protein to be visualized by staining (usually 1–5 μg), is applied to a parallel gradient and it is processed exactly as the protein of interest. The sedimentation positions of the marker proteins are determined by SDS-PAGE and subsequent staining with Coomassie brilliant blue or silver.

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